University of Alberta

The Role of Arginine and Arginase in *Pinus taeda* L. Early Seedling Growth

by

Janice Elizabeth King

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biological Sciences

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ABSTRACT

In loblolly pine (*Pinus taeda* L.), storage proteins in the megagametophyte make up 70% of the total protein in the mature seed. As the embryo develops into a seedling, amino acids arising from the breakdown of these storage proteins are exported to the seedling to support growth and development. The objectives of this thesis were to develop a model describing the breakdown of storage proteins in the megagametophyte and the fate of amino acids arising from the breakdown of these proteins, as well as to identify factors involved in the regulation of these processes.

A number of experiments were conducted to demonstrate the importance of the megagametophyte in providing the products of storage protein breakdown to the developing seedling. Although the megagametophyte is largely autonomous in its ability to elicit storage protein breakdown and amino acid export, the seedling may be able to exert some control over the supply of amino acids that it receives from the megagametophyte. Elevated levels of arginine in the megagametophyte might be one mechanism by which storage protein breakdown is negatively regulated.

Arginine is the primary amino acid comprising the major seed storage proteins in the megagametophyte of loblolly pine, and is also a major component of the free amino acid pools in both the megagametophyte and the seedling during early seedling growth. A major route of arginine assimilation into metabolic and biosynthetic pathways is via arginase, an enzyme converting arginine to ornithine and urea. Arginase activity is found mainly in the seedling cotyledons, and increases concomitantly with the free arginine content of the seedling. Seedling development is impaired when assimilation of urea

generated by arginase-mediated arginine breakdown is blocked, demonstrating that nitrogen derived from this pathway is important for early seedling growth. The megagametophyte is required for both induction and maintenance of arginase activity in the seedling. Arginase activity appears to be positively regulated by arginine, and negatively regulated by an accumulation of urea. Arginase was purified from loblolly pine seedlings, and anti-arginase antibodies elicited to the protein. These antibodies will be an important tool for further studies on the regulation of this enzyme.

To My Grandparents

J. Tait Galland (1911 - 1993)

Elinor L. Galland (1916 - 1997)

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LIST OF ABBREVIATIONS

ABA	abscisic acid
arg	arginine
ala	alanine
asn	asparagine
asp	aspartate
asx	asparagine + aspartate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
µCi	microCurie
CoA	coenzyme A
cys	cysteine
kD	kilodalton
DAI_2	days after imbibition at 2°C
DAI_{30}	days after imbibition at 30°C
dpm	decays per minute
EDTA	ethylenediaminetetraacetic acid
FPLC	fast protein liquid chromatography
g	gravity
GF/C	glass fibre circles
gln	glutamine

glu	glutamate
glx	glutamine + glutamate
gly	glycine
his	histidine
HPLC	high pressure liquid chromatography
IEF	isoelectric focusing
ile	isoleucine
K_m	Michaelis constant
leu	leucine
lys	lysine
MES	2-(N-morpholino)ethanesulfonic acid
met	methionine
MWCO	molecular weight cutoff
NBT	nitro blue tetrazolium chloride
NMWL	nominal molecular weight limit
PAGE	polyacrylamide gel electrophoresis
phe	phenylalanine
PITC	phenylisothiocyanate
PPD	phenylphosphorodiamidate
PMSF	phenylmethylsulfonylfluoride
pro	proline
rpm	revolutions per minute

S	Svedberg
SDS	sodium dodecyl sulfate
ser	serine
SE	standard error
TAG	triacylglycerol
TCA	trichloroacetic acid
TCA cycle	tricarboxylic acid cycle
TEMED	N, N, N', N'-tetramethylethylenediamine
thr	threonine
trp	tryptophan
V	volts
val	valine

1. INTRODUCTION

1.1 Loblolly Pine

The natural range of loblolly pine (*Pinus taeda* L.)[1] extends from West Texas to the Atlantic coast, and from New Jersey to Northern Florida (Fig. 1.1) (Fowells, 1965). This region has become the largest supplier of wood products in the U.S., attributable in part to the high productivity of the region's forest lands, and the implementation of intensive forest management practices (Zinkhan and Mercer, 1997). In 1992, there were 9.3 million ha of intensively managed pine plantations in the southeastern United States, representing approximately 15.7% of the forest land base, and 7.4% of the total land base of the region (Zinkhan and Mercer, 1997). Loblolly pine is the principal commercially-grown forest tree in the southeastern U.S., primarily due to its fast growth rate (Carey, 1992), ability to grow in pure stands, and versatility for use in a wide array of forest products (Fowells, 1965). Nearly 1 billion bare-root loblolly pine seedlings were produced for reforestation programmes in 1980, representing 75% of the seedlings produced in the southeastern forest region, and 60% of all bare-root nursery stock produced in the U.S. (Boyer and South, 1984). Given the increasing shift towards loblolly pine as the preferred forestry species in the southeast (Boyer and South, 1984), these numbers are likely to be higher today.

The same characteristics that make loblolly pine an attractive species for intensive forestry in the U.S. have also led to its increasing use in other countries. For example,

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[1] At the first mention of a species, both the common name and the scientific name will be given. Where feasible, the common name will be used in subsequent references to the species.

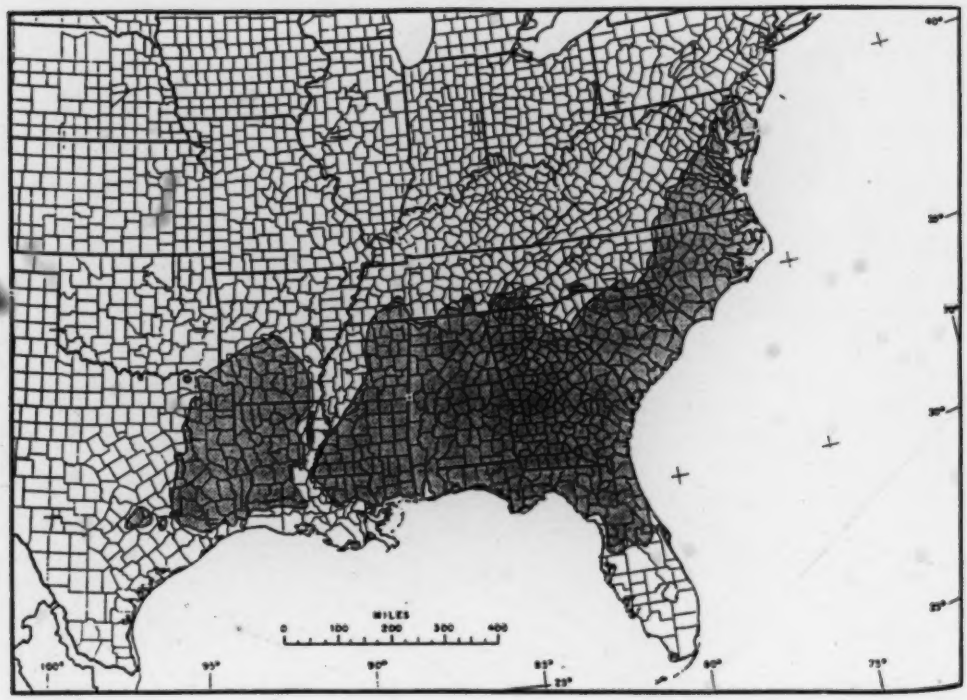

FIGURE 1.1 Natural distribution of loblolly pine (*Pinus taeda* L.). From Fowells (1965).

China plants approximately 100 000 hectares of slash pine and loblolly pine each year. This requires approximately 66 000 kg of seed annually (UNDP Report, 1997). Loblolly pine is also a commercially important species in South Africa (Hagedorn, 1994) and in South America (Becwar and Pullman, 1995).

Because of loblolly pine's fast growth and abundant litter production, this species is also widely used in habitat remediation, e.g. mine reclamation or soil stabilization (Carey, 1992).

1.2 Structure of the Mature Loblolly Pine Seed

The diploid embryo of a loblolly pine seed is enclosed within a haploid, maternally derived tissue referred to as the female gametophyte or megagametophyte (Fig. 1.2). The embryo lies within the corrosion cavity that forms in the megagametophyte during seed development. The megagametophyte and the embryo are not symplastically connected, but upon imbibition, the tissues swell so that there is close contact between the two seed parts. The megagametophyte is enveloped by the megaspore wall (Gifford and Foster, 1989), and its micropylar end is covered by the lipophilic, tannin-rich nucellar cap (Tillman-Sutela and Kauppi, 1995a, 1995b). The entire seed is encased within a thick, hard seed coat, or testa, which consists primarily of the sclerotesta (Berlyn, 1972; Gifford and Foster, 1989). Between the seed coat and the megagametophyte is a thin papery layer: the identity of this layer is not clear, but evidence from Scots pine (*Pinus sylvestris* L.) indicates that it may include remnants of the nucellus (Tillman-Sutela and Kauppi, 1995b).

FIGURE 1.2 Light micrograph of a median longitudinal section through a mature seed of loblolly pine, with the testa and papery layer removed. The diploid embryo is enclosed within the haploid, maternally derived megagametophyte. Scale: 10 mm = 500 µm. From Stone and Gifford (1997).

1.3 Seedling Growth and Development

The development of a seedling from a seed can be demarcated into three phases: dormancy-breaking, germination, and early seedling growth. These phases of growth and development are described below.

1.3.1 Dormancy-Breaking

In species that exhibit seed dormancy, some physical or molecular block exists that prevents an intact, viable seed from germinating when it is exposed to favourable environmental conditions (Bewley, 1997). Primary dormancy is acquired by seeds prior to being shed from the plant, while secondary dormancy may be acquired following seed dispersal. A number of mechanisms appear to have evolved to induce and maintain seed dormancy, which may be divided into two general categories: coat-imposed dormancy and embryo dormancy (Bewley, 1997). Coat-imposed dormancy is defined as dormancy exerted upon the embryo by one or more of the surrounding tissues in the seed. If the constraining tissues are removed, the embryo readily germinates. The tissue may exert mechanical resistance to radicle emergence, interfere with water uptake or gas exchange, provide a source of compounds that inhibit germination, or prevent such chemicals from exiting the embryo (Bewley and Black, 1994). In embryo dormancy, the factor(s) preventing dormancy reside within the embryo itself. ABA has been implicated as a dormancy-imposing factor in several species (Bewley, 1997). Embryo dormancy may also result when the seed is shed while the embryo is still physiologically immature. The embryos of such seeds must mature after dispersal before they are able to germinate (Kozlowski and Pallardy, 1997). Some species exhibit varying degrees of both coat-imposed and embryo dormancy (Kozlowski and Pallardy, 1997).

Dormancy-breaking is the phase of development during which the factor(s) imposing dormancy are alleviated, allowing the seeds to germinate when placed in the appropriate conditions. In seeds of some species, dormancy is lost during storage in dry conditions. This process is known as afterripening. Dormancy is alleviated in seeds of other species by stratification, defined as incubation of seeds with moisture at low temperatures or alternating high and low temperatures. Chemicals, mechanical scarification, and heat may also be used to break dormancy (Kozlowski and Pallardy, 1997).

The mechanisms by which these empirical methods of dormancy-breaking work to overcome barriers to germination are generally unknown (Kozlowski and Pallardy, 1997). The appearance and disappearance of both proteins and mRNAs coinciding with dormancy-breaking in wheat (*Triticum aestivum* L.) (Morris *et al.*, 1991); cheat grass (*Bromus secalinas*) (Goldmark *et al.*, 1992), sugar maple (*Acer saccharum* Marsh.) (Hance and Bevington, 1992), Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) (Taylor *et al.*, 1993; Taylor and Davies, 1995; Jarvis *et al.*, 1996, 1997), loblolly pine (Schneider and Gifford, 1994; Mullen *et al.*, 1996), and wild oat (*Avena fatua* L.) (Li and Foley, 1996), suggest that changes in gene expression may be involved in the maintenance and/or breakage of dormancy. However, these studies only provide correlative evidence for the participation of specific gene products in overcoming dormancy. For species in which tissues surrounding the embryo pose a physical barrier to germination, activation of cell wall degrading enzymes such as endo-β-mannanase and hemicellulases may act to loosen constraining tissues near the radicle tip, permitting the radicle to penetrate the tissues (Nonogaki and Morohashi, 1996; Downie *et al.*, 1997). Light is known to play an

important role in dormancy-breaking of many species, often as an activator of a phytochrome-mediated cascade of events (Bewley and Black, 1994). Temperature may act to alter the sensitivity of the phytochrome response to light (Karssen, 1995). Exposure to certain environmental conditions may also increase the sensitivity of the seed to germination-stimulating factors like gibberellins and nitrate (Karssen, 1995).

1.3.2 Germination

Germination is defined as the period that begins upon imbibition of the mature, desiccated seed, and ends when a portion of the seedling, usually the radicle, has protruded beyond the edge of the seed coat (Bewley, 1997). During the initial phase of water uptake, existing cellular structures are hydrated, and metabolism resumes (Obroucheva and Antipova, 1997). Biochemical events that take place during these first few hours of imbibition are dependent upon organelles and enzymes that were present in the desiccated seed (Bewley and Black, 1994), and upon proteins synthesized from stored mRNAs on pre-existing ribosomes (Beltrán-Peña *et al.*, 1995). Within a few hours after the onset of imbibition, cells begin *de novo* synthesis of enzymes and cellular structures necessary for the processes involved in radicle emergence and early growth of the seedling (Obroucheva and Antipova, 1997).

Extension of the radicle appears to result mainly from increased turgor in the cells comprising the radicle; cell division may or may not occur during the initial extension required for radicle protrusion. Extension of the radicle cells may result from solute accumulation, possibly enhanced by breakdown of reserves within these cells. Solute accumulation is likely concomitant with loosening of the walls of the cells. Seed tissues

constraining the tip of the radicle may also be weakened by enzymatic degradation, as described in Section 1.3.1 (Bewley, 1997).

In this thesis, loblolly pine seed dormancy was overcome by seed stratification - i.e. chilling with moisture - prior to placing the seeds in conditions favorable for germination. During stratification, water imbibition occurs at temperatures that do not permit visible germination to occur. *De novo* mRNA and protein synthesis, indicators of metabolic activity, are evident during the course of loblolly pine seed stratification (Schneider and Gifford, 1994; Mullen *et al.*, 1996). However, many events associated with seed hydration, such as protein synthesis from mRNAs that have been stored in the mature desiccated seed, are defined as germination-associated processes. Therefore, in the experimental system used in this thesis, there is temporal overlap of dormancy-breaking events with some germination-associated events. For the purposes of this thesis, germination will be considered as the period of time starting when the seeds are transferred from dormancy-breaking conditions to germination conditions, and finishing when the radicle has extended beyond the edge of the seed coat.

1.3.3 Early Seedling Growth

Following radicle emergence, the shoot and root apices of the embryo begin to produce the cells that will differentiate to form the organs of the mature plant. Elongation and differentiation of the hypocotyl also occurs (Gifford and Foster, 1989). Substantial *de novo* mRNA and protein synthesis accompanies the growth and differentiation of these cells (e.g. Dure *et al.*, 1981; Datta *et al.*, 1987; Sundås *et al.*, 1992; Groome *et al.*, 1991; Mullen *et al.*, 1996). The seedling also synthesizes the components required for photosynthesis, in preparation for autotrophy. In general, pine species develop the

capacity to photosynthesize soon after emergence of the seedling from the seedbed (Kozlowski and Pallardy, 1997).

The catabolism of starch, lipid, and protein reserves that are accumulated by the seed during embryogenesis supplies the nutrients that drive the metabolic and biosynthetic pathways required to support seedling growth and development (Bewley and Black, 1994). In most species, some or most of the seed storage reserves are located within specialized storage tissues, such as the cotyledons, perisperm, endosperm, or megagametophyte (Bewley and Black, 1994). Complex polysaccharides in the cell walls of the storage tissue, such as mannans and xyloglucans, may also be classified as a storage reserve (Lopes and Larkins, 1993). In addition to these storage reserves, seeds contain mineral reserves, which are often chelated to phytic acid (Lott *et al.*, 1995).

In this thesis, early seedling growth is defined as the period of development during which the seedling is receiving products of storage reserve breakdown from the storage tissue. It has been demonstrated in *Zea mays* that utilization of nutrients from the surrounding medium, such as nitrate, is inhibited while the products of storage reserve breakdown are available to support seedling growth (reviewed in Oaks, 1997). Elevated levels of free amino acids, accumulated as a result of storage reserve breakdown, appear to inhibit extracellular nitrate uptake (Padgett and Leonard, 1996), as well as to repress transcription and inhibit activity of nitrate reductase to some extent (Li *et al.*, 1995; Sivasankar and Oaks, 1995; Sivasankar *et al.*, 1997, Oaks, 1997).

1.4 Seed Storage Reserve Breakdown, Transport, and Utilization

Proteins and lipids constitute the bulk of the storage reserves in seeds of most of the Pinaceae species examined to date, including Douglas-fir (Ching, 1966; Owens *et al.*,

1993), Scots pine (Simola, 1974), Norway spruce (*Picea abies* [L.] Karst = *Picea excelsa* Lam.) (De Carli *et al.*, 1987; Hakman, 1993), Italian stone pine (*Pinus pinea* L.) (Gori, 1979), silver fir (*Abies alba* Mill.) (Kovac and Kregar, 1989), and loblolly pine (Groome *et al.*, 1991). Therefore, the discussion of reserve breakdown, transport, and utilization will be limited to storage lipids and proteins, with an emphasis on findings in conifer species.

1.4.1 Triacylglycerol Reserves

1.4.1.1 Triacylglycerol Reserve Catabolism

Triacylglycerols (TAGs) are the major form of lipid reserves in the seeds of most species (Ching, 1972; Trelease and Doman, 1984). During seed development, TAGs accumulate in spherical, half-unit membrane-bounded lipid bodies (Trelease and Doman, 1984). When germination commences, most TAGs are converted to sugars for use by the developing seedling (Trelease and Doman, 1994). The first step in the catabolic pathway is the lipase-mediated breakdown of TAGs to glycerol and fatty acids. Several distinct lipases appear to be involved in complete fatty acid release from TAGs in the species studied to date, and may be associated with either the lipid bodies or the glyoxysomes (Galliard, 1980). TAGs are then metabolized to acetyl CoA units via β-oxidation. Free fatty acids do not accumulate in the tissues where they are generated, suggesting that they quickly enter β-oxidation (Galliard, 1980). The acetyl CoA enters the glyoxylate cycle, generating succinate and malate that are used to synthesize phosphoenolpyruvate (Trelease and Doman, 1984). The phosphoenolpyruvate then enters gluconeogenesis, resulting in the synthesis of sugars for use by the growing seedling (Ching, 1972; Trelease and Doman, 1984). Most of the glycerol arising from the action of lipases on TAGs is

also channeled into gluconeogenesis by conversion to glyceraldehyde-3-phosphate (Bewley and Black, 1994).

A role for these biochemical pathways in storage lipid catabolism has been established in conifer megagametophytes. Acid lipases have been characterized in Douglas-fir (Ching, 1968) and pinyon pine (*Pinus edulis* Engelm.) (Hammer and Murphy, 1994), and were demonstrated to be associated with lipid bodies, indicating their probable role in storage TAG breakdown. Lipases have also been described for Scots pine (Nyman, 1965), radiata pine (*Pinus radiata* D. Don) (Kao and Rowan, 1970), and *Abies alba* (Kovac and Wrischer, 1984). Enzymes of the glyoxylate cycle have been described in several conifer species, including Italian stone pine (Firenzuoli *et al.*, 1968), *Abies alba* (Kovac and Wrischer, 1984), ponderosa pine (*Pinus ponderosa* Laws.) (Ching, 1970; Bilderback, 1974; Murray and Adams, 1980), sugar pine (*Pinus lambertiana* Dougl.) (Noland and Murphy, 1984), and loblolly pine (Mullen and Gifford, 1993, 1995a, 1995b, 1997). Enzymes of β-oxidation, gluconeogenesis, and other steps involved in TAG catabolism have also been examined in conifers (Pitel and Cheliak, 1988; Gifford, unpublished), although in less detail than lipases and glyoxylate cycle enzymes.

In contrast to the megagametophyte, developing seedlings of the conifer species studied to date demonstrate low to negligible activity of the glyoxylate cycle enzyme isocitrate lyase (Ching, 1970; Kovac and Wrischer, 1984; Noland and Murphy, 1984; Groome, 1991). This lack of activity has been interpreted to mean that the glyoxylate cycle may not be fully functional in this tissue (Kovac and Wrischer, 1984; Hammer and Murphy, 1994), even though the mature conifer embryo generally contains appreciable quantities of storage lipids (Durzan *et al.*, 1971; Simola, 1974; Kovac and Vardjan, 1981;

De Carli *et al.*, 1987; Hakman, 1993; Stone and Gifford, unpublished). This has lead to the hypothesis that TAG reserves in the developing conifer seedling may be metabolized via pathways other than the glyoxylate cycle and gluconeogenesis (Simola, 1974; Kovac and Wrischer, 1984; Hammer and Murphy, 1994). To date, there is no experimental evidence to support this hypothesis in conifers. However, in some angiosperm species, the acetyl-CoA that results from β-oxidation of free fatty acids can be used to generate amino acids via select reactions of the glyoxylate and TCA cycle (Bewley and Black, 1994). It is conceivable, then, that acetyl-CoA arising from TAGs catabolism may be channeled into other metabolic pathways in the seedlings of some conifer species. These alternative metabolic pathways could operate in addition to - or perhaps exclusive to - the conversion of TAGs to sugars via the glyoxlyate and gluconeogenic pathways.

1.4.1.2 Transport and Utilization of the Products of Triacylglycerol Catabolism

In general, sucrose is the major product of lipid catabolism in angiosperm species (Trelease and Doman, 1984). In castor bean (*Ricinus communis* L.), nearly all of the hexose sugars arising from lipid catabolism are converted to sucrose for export from the endosperm to the seedling (Canvin and Beevers, 1961; Kriedemann and Beevers, 1967a, 1967b). There is some evidence that a sucrose-H^+ co-transporter on the surface of the cotyledons might facilitate sucrose uptake (Lucas and Madore, 1988). Fructose and glucose also appear to be exported from the endosperm and taken up by the seedling, although in much smaller quantities (Kriedemann and Beevers, 1967b). Ching (1970) demonstrated in ponderosa pine that sugars are exported from the megagametophyte during TAG breakdown, but these sugars were not identified. Preliminary evidence

obtained in loblolly pine indicates that these sugars may be primarily glucose and fructose, rather than sucrose (Stone and Gifford, unpublished).

In castor bean, sucrose delivered from the endosperm to the cotyledons is transported to the growing axis of the seedling via the phloem (Lucas and Madore, 1988). The sucrose must then be converted to glucose and fructose by invertase or to UDP-glucose and fructose by sucrose synthase (sucrose synthetase) in the seedling axis before it can be used for respiration or as precursors for biosynthetic pathways (Bewley and Black, 1994; Thomas and Rodriguez, 1994). Increasing invertase activity was demonstrated during radiata pine early seedling growth (Kao and Rowan, 1970). An increase in the activity of soluble acid invertase in developing seedlings was also reported in sugar pine (Murphy and Hammer, 1988) and in pinyon pine (Murphy *et al.*, 1992). Based on the correlation of soluble acid invertase activity with early seedling growth, Murphy *et al.* (1992) proposed that this enzyme was likely to be involved in providing substrates for metabolic and biosynthetic pathways. Considerable sucrose synthase activity was also shown in seedlings of sugar pine and pinyon pine during the first part of early seedling growth (Murphy and Hammer, 1988; Murphy *et al.*, 1992), and was correlated with the deposition of starch in the cotyledons and the hypocotyl (Murphy and Hammer, 1994). [^{14}C]-sucrose labelling experiments confirmed that a significant percentage of the sucrose was being partitioned into starch (Murphy and Hammer, 1994). The authors suggested that sucrose synthase may be involved in synthesizing starch as a transient storage pool for sucrose received by the seedling from the megagametophyte that was not immediately required to support metabolic and biosynthetic pathways (Murphy and Hammer, 1994). Histochemical studies with other conifer species have also shown starch accumulation in

the seedling during early growth (Ching, 1966; Durzan *et al.*, 1971; Simola, 1974; De Carli *et al.*, 1987; Kovac and Kregar, 1989), lending some support to the hypothesis. The hypothesis is congruent with those of Trelease and Doman (1984) and Chapman and Galleschi (1985), who proposed that starch accumulation in the cotyledons of angiosperm species acts as a sink for sugars arising from lipid catabolism, thereby avoiding accumulation of metabolites that might inhibit enzymes in the lipid catabolic pathway.

1.4.2 Protein Reserves

1.4.2.1 Classification and Characterization of Seed Storage Proteins

Seed storage proteins are sequestered within single-membrane bounded storage protein vacuoles (protein bodies) (Lott, 1980). The classification of seed storage proteins is often based on the differential solubility scheme proposed by Osborne (1918).

- albumins: soluble in water and dilute buffers at neutral pH
- globulins: insoluble in water, but soluble in salt solutions
- prolamins: soluble in aqueous alcohols
- glutelins: soluble in dilute solutions of acid or base

Within these broad classes, storage proteins can further be characterized by the sedimentation coefficient of the holoprotein (Shewry *et al.*, 1995).

The albumins are found in most dicotyledonous species (Shewry *et al.*, 1995). Most albumins, referred to as 2S albumins, are small, globular, heterodimeric proteins, consisting of a large and a small subunit cleaved from a single precursor polypeptide, and linked by disulfide bridges (Shewry *et al.*, 1995). Some 2S storage proteins exhibit globulin-like solubilities, but share structural and sequence homology with the 2S

albumins. Thus, the 2S globulins and 2S albumins are often considered to represent a single class of 2S proteins (Allona *et al.*, 1994a).

Globulins are present to varying degrees in the seeds of most higher plants studied to date (Higgins, 1984; Shewry *et al.*, 1995). This class of storage proteins is usually divided into two subclasses: the 7S globulins or vicilin-like proteins, and the 11S globulins or legumin-like proteins. The 11S legumin-like proteins are hexameric, with each of the six subunits consisting of an acidic polypeptide (33 to 42 kD) linked to a basic polypeptide (19 to 23 kD) via one disulfide bridge (Bewley and Black, 1994; Shewry *et al.*, 1995). In general, the 7S vicilin-like proteins are trimeric holoproteins with non-identical subunits that do not form disulfide bridges (Shewry *et al.*, 1995). Subunit heterogeneity in the 7S vicilin-like proteins arises from differential cleavage of the precursor polypeptides, as well as from variability in post-translational glycosylation (Shrewry *et al.*, 1995).

Prolamins are the major storage proteins in most of the Gramineae, but are absent in other plant families (Shotwell and Larkins, 1989; Shewry *et al.*, 1995). There are three subclasses of prolamins: the S-rich proteins, the S-poor proteins, and the high molecular weight proteins. The S-rich proteins may be monomeric or polymeric, and can exhibit extensive disulfide bridging. They are characterized by repetitive domains consisting of proline- and glutamine-rich repeated sequences. The S-poor proteins generally are not oligomeric proteins due to a lack of cysteine residues for disulfide bridging. The S-poor proteins examined to date have consisted almost solely of repeated sequences of an eight amino acid motif that, like the S-rich proteins, is rich in proline and glutamine. The high

molecular weight prolamins also contain extensive repeated sequences, and share significant sequence homology with the S-rich prolamins (Shewry *et al.*, 1995).

Glutelins are also found in members of the Gramineae (Larkins, 1981; Shewry *et al.*, 1995). This class of storage proteins has been less studied than the other classes of storage proteins, due to the difficulty of extracting the proteins (Larkins, 1981). Glutelins in wheat, referred to as glutenin, consist of high molecular weight and low molecular weight subunits (Casey and Domoney, 1987). The glutenin holoprotein is a highly insoluble aggregate of approximately 17 polypeptides held together by hydrophobic interactions and disulfide bridging (Larkins, 1981). High molecular weight glutenin genes analyzed to date exhibit an extensive region coding for multiple tandem repeats of a 6 to 12 amino acid motif rich in glycine, as well as glutamine and proline (Rafalski *et al.*, 1986; Shotwell and Larkins, 1989). Based on conservation of amino acid sequences and structural features with other prolamins, glutenins are now considered to be members of the prolamin superfamily (Shotwell and Larkins, 1989; Shewry, 1993; Shewry *et al.*, 1995).

Wheat glutenin and the analogous proteins in maize and barley are unusual in that the mature proteins may accumulate outside storage protein vacuoles (Larkins, 1981; Bewley and Black, 1994). The aggregation of these large proteins in the lumen of the ER during processing is thought to cause the organellar membrane to rupture, releasing them into the cytosol (Larkins, 1981; Bewley and Black, 1994).

An additional class of storage proteins is only solubilized by dissociating agents such as urea, or by boiling in the presence of detergents such as SDS (Bewley and Black, 1994). Storage proteins with these solubility characteristics are often referred to as

crystalloid proteins, based upon the nomenclature developed by Gifford *et al.* (1982) for castor bean. Jensen and Berthold (1989) and Bewley and Black (1994) suggest that the crystalloid proteins of castor bean are homologous to the 11S globulins.

1.4.2.2 Seed Storage Proteins in Conifers

A number of studies describing seed storage proteins in conifer species have appeared in the last decade. The major seed storage proteins in several *Pinus* species were originally referred to as crystalloids by Gifford (1988), based on similarities of solubility and electrophoretic characteristics with the major seed storage proteins in castor bean. The term "crystalloid" was also used to describe the major seed storage proteins in white spruce (*Picea glauca* [Moench] Voss.) (Gifford and Tolley, 1989; Misra and Green, 1990), Engelmann spruce (*Picea engelmanii* Parry ex Engelm.), Sitka spruce (*Picea sitchensis* [Bong.] Carr.), black spruce (*Picea mariana* Mill.), (Misra and Green, 1990), and Douglas-fir (Green *et al.*, 1991a). However, Jensen and Lixue (1991) and Allona *et al.* (1994b) classify the seed storage proteins from these species, as well as those from other *Pinus* and *Picea* species, as 11S legumin-like proteins. Legumin-like proteins have also been reported in Norway spruce (Hakman, 1993), dawn redwood (*Metasequoia glyptostroboides* Hu & Cheng) (Häger and Dank, 1996), *Cedrus deodara* (Roxb.) G. Don, tamarack (*Larix laricina* [Du Roi] K. Koch), and Siberian larch (*Larix sibirica* Ledeb.) (Jensen and Lixue, 1991). Storage protein cDNA clones isolated from Douglas-fir (Leal and Misra, 1993), interior spruce (*Picea glauca/engelmanii* complex) (Flinn *et al.*, 1993), white spruce (Leal *et al.*, 1995), eastern white pine (*Pinus strobus* L.) (Baker *et al.*, 1996), and incense cedar (*Calocedrus decurrens* [Torr.] Florin) (Häger and Dank, 1996) show homology to the legumin-like globulins. N-terminal sequence analysis,

serological cross-reactivity, and holoprotein structure indicated that the major seed storage proteins in maritime pine (*Pinus pinaster* Ait.) also share homology with the 11S globulins, even though the solubility characteristics of these proteins imply that they be classified as glutelins (Allona *et al.*, 1992).

The criteria of solubility and electrophoretic patterns were used to describe vicilin-like proteins in maritime pine (Allona *et al.*, 1992, 1994c), Norway spruce, *Abies alba*, *Taxus baccata* L., *Biota orientalis* (L.) Endl., *Chamaecyparis lawsoniana* (Murr.) Parl, and *Cupressus arizonica* Green (Allona *et al.*, 1994b). A cDNA encoding a vicilin-like protein has also been isolated and characterized in interior spruce (Newton *et al.*, 1992).

Electrophoretic and solubility characteristics were also used to identify albumins as the major seed storage proteins in yellow cypress (*Chamaecyparis nootkatensis* [D. Don] Spach.) (Kurz *et al.*, 1994) and in several species of *Abies* (Jensen and Lixue, 1991). Albumins have also been reported in interior spruce (Flinn *et al.*, 1991, 1993) and in maritime pine (Allona *et al.*, 1992). Maritime pine contained 2S globulins, as well (Allona *et al.*, 1994a).

1.4.2.3 Amino Acid Composition of Seed Storage Proteins

Seed storage proteins are often rich in the amide amino acids glutamine and asparagine (Van Etten *et al.*, 1963; Shotwell and Larkins, 1989), but also may be rich in arginine (Van Etten *et al.*, 1963; Derbyshire *et al.*, 1976; Higgins, 1984). All three of these amino acids have a high nitrogen-to-carbon ratio, making them particularly suited for storage of N (Bray, 1983). Arginine appears to be a preferred form of stored nitrogen in tree species (see references in Durzan and Steward, 1983). Amino acid analysis of maritime pine storage proteins (Allona *et al.*, 1992, 1994a, 1994c), and deduced amino

acid sequences of storage protein cDNAs from interior spruce (Newton *et al.*, 1992), Douglas-fir (Leal and Misra, 1993), and incense cedar (Häger and Dank, 1996) indicate that arginine is a major constituent of the storage proteins in these conifer species.

1.4.2.4 Protein Reserve Breakdown During Post-Embryonic Growth

Several classes of proteolytic enzymes, referred to as proteinases or peptidases, are involved in storage protein breakdown. Peptidases may be classified either as endopeptidases, which cleave internal peptide bonds, or exopeptidases, which sequentially cleave the terminal amino acids off of the peptide. Exopeptidases may act at the amino end of the polypeptide (aminopeptidases), or may cleave amino acids from the carboxyl terminal (carboxypeptidases) (Bewley and Black, 1994). In general, the breakdown of storage proteins to peptides occurs within protein bodies, while the resulting peptides are degraded to amino acids in the cytoplasm (Bewley and Black, 1994).

Storage protein mobilization requires the sequential activities of a suite of these proteolytic enzymes to break down the proteins to their constituent amino acids and/or small peptides. In maize, in which zeins are the major storage proteins, at least 17 endopeptidases are active during early seedling growth. These enzymes can be divided into four distinct groups: 1. endopeptidases that are present in the dry seed, but decline soon after imbibition, 2. cysteine endopeptidases with an affinity for γ-zein, that appear shortly after radicle emergence, but disappear after two days, 3. endopeptidases, capable of breaking down both γ-zein and α-zein, that appear shortly after radicle emergence and maintain high activity until storage protein breakdown is completed, and 4. endopeptidases specific for α-zein that appear only during the final stages of storage protein mobilization (Mitsuhashi and Oaks, 1994). Similar patterns of endopeptidase

activities were also seen in wheat, in which 20 endopeptidases were detected (Domínguez and Cejudo, 1995).

In some species, e.g. wheat and barley, the storage proteins are subjected to enzymatic modifications prior to breakdown. These modifications appear to render the proteins more susceptible to proteolytic cleavage. It is unclear whether seed storage proteins in other species undergo a similar modification (Bewley and Black, 1994).

Although patterns of storage protein breakdown have been described in several conifer species, including lodgepole pine (Lammer and Gifford, 1989), white spruce (Gifford and Tolley, 1989), interior spruce (Flinn *et al.*, 1991), Douglas-fir (Green *et al.*, 1991a), loblolly pine (Groome *et al.*, 1991; Stone and Gifford, 1997), and yellow cypress (Kurz *et al.*, 1994), there has not yet been a comprehensive study of the step-wise processes required to elicit protein reserve breakdown in conifers. Proteolytic enzymes active during early seedling growth have been partially characterized in Italian stone pine (Guitton, 1964), Scots pine (Salmia *et al.*, 1978; Salmia 1981a, 1981b; Salmia and Mikola, 1975, 1976), sugar pine (Noland and Murphy, 1986), and lodgepole pine (*Pinus contorta* Dougl.) (Gifford *et al.*, 1989). A cDNA encoding a putative cysteine protease has been characterized in Douglas-fir. The pattern of expression suggests that the protease encoded for by the cDNA could participate in storage protein breakdown (Tranbarger and Misra, 1996).

1.4.2.5 Transport and Utilization of the Products of Protein Reserve Breakdown

In many dicotyledonous species, the majority of the amino acids that arise from storage protein breakdown are metabolized to another form, often glutamine or asparagine, before they are transported to the axis (Stewart and Beevers, 1967; Dilworth

and Dure, 1978; Kern and Chrispeels, 1978; Higgins and Payne, 1980; Bray, 1983; Lea and Miflin, 1989; Bewley and Black, 1994). Transport to the axis likely occurs via the phloem (Robinson and Beevers, 1981; Murray, 1984; Bewley and Black, 1994), although other mechanisms of amino acid transport could be important in some species (Higgins and Payne, 1980). The transported compounds are then assimilated into metabolic and biosynthetic pathways in the developing seedling axis (Bewley and Black, 1994). Peas are unusual in that amino acids arising from storage protein breakdown in the cotyledons are converted to homoserine, a non-protein amino acid, for transport to the axis (Larson and Beevers, 1965).

Most of the free amino acid products of storage protein breakdown in the endosperm of castor bean are converted to glutamine for transport to the seedling, although some amino acids, such as valine, generally are not metabolized prior to transport (Stewart and Beevers, 1967; Robinson and Beevers, 1981). Amino acids exported by the endosperm appear to be taken up by the cotyledons via one major, broad-ranged amino acid transport system (Robinson and Beevers, 1981). In addition, some free amino acids arising from storage protein breakdown in castor bean - particularly aspartate, glutamate, alanine, glycine, serine, and leucine - are deaminated in the endosperm, and the carbon skeletons channeled into gluconeogenesis. The resulting sucrose is then transported from the endosperm to the developing seedling (Stewart and Beevers, 1967).

In cereals, storage proteins in the endosperm are broken down to a mixture of small peptides and amino acids, which are exported to the scutellum of the seedling without prior metabolic interconversions (Higgins and Payne, 1980; Higgins and Payne, 1981; Sopanen and Väisänen, 1985). Several classes of amino acid transporters, some of

which exhibit amino acid specificity, have been characterized in barley, wheat, rice, and maize scutella (Mikola and Mikola, 1980; Sopanen *et al.*, 1980; Sopanen and Väisänen, 1985; Väisänen and Sopanen, 1986; Salmenkallio and Sopanen, 1989). Transport of small peptides has also been characterized in the scutella of barley and other cereals, and appears to be a function of a single non-specific peptide transporter (Higgins and Payne, 1978; Sopanen *et al.*, 1977; Sopanen, 1979; Walker Smith and Payne, 1983). Once taken up, the peptides are broken down to amino acids by peptidases in the scutellum (Sopanen *et al.*, 1978). Free amino acids are transported without metabolic interconversion from the scutellum to the growing coleoptile and roots, where they are integrated into metabolic and biosynthetic pathways (Higgins and Payne, 1980).

Few studies have examined the fate of amino acids arising from seed storage protein breakdown in conifer species. To date, nothing has been published on the transport of amino acids from the megagametophyte to the seedling during early seedling growth, although movement of the amino acid products of storage protein breakdown from the megagametophyte to the seedling is a commonly-accepted paradigm (e.g. Durzan *et al.*, 1971; Bilderback, 1974; Simola, 1974; Carpita *et al.*, 1983; De Carli *et al.*, 1987). The metabolism of only a few protein amino acids by the seedling have been described. The breakdown of arginine by arginase during early seedling growth has been examined in Italian stone pine (Guitton, 1964) and, to a lesser extent, in maritime pine, *Abies pectinata* Lamk. D.C. (Guitton, 1957) and Aleppo pine (*Pinus halepensis* Mill.) (Citharel and Citharel, 1975). Because of the focus on arginine in this thesis, the metabolism of this amino acid is discussed in further detail in Section 1.5. Glutamine synthetase, which produces glutamine from glutamate and ammonium and is considered to play a pivotal role

in the assimilation of amino acids into metabolic and biosynthetic pathways, has been examined in maritime pine. The cytosolic form of the enzyme was found to be present in the mature unimbibed embryo, and increased during early seedling growth, mainly in the cotyledons (Cánovas *et al.*, 1991). A cDNA encoding a cytosolic glutamine synthetase has been isolated from Scots pine seedlings by the same group of researchers (Cantón *et al.*, 1993). However, the role that this enzyme plays in the utilization of the amino acid products of storage protein breakdown in conifers has not been addressed. The metabolism of proline in jack pine seedlings has also been investigated using radiolabelling studies (Durzan and Ramaiah, 1971).

1.4.3 Control of Reserve Mobilization

Interactions between the developing seedling axis and the storage tissue are important in controlling reserve mobilization in many dicot and monocot species (see Murray, 1984; Bewley and Black, 1994). There are two major mechanisms by which the seedling axis may exert control over reserve breakdown: 1. the axis may act as a sink for the products of reserve breakdown, and 2. the axis may secrete signal molecules that regulate reserve breakdown.

In some species, such as cucumber (*Cucumis sativus* L.), storage tissues accumulate the products of reserve breakdown when cultured in the absence of the seedling axis, indicating that the axis provides a sink for the movement of these nutrients out of the storage tissue (Davies and Chapman, 1979a; Murray, 1984). Elevated levels of reserve-derived metabolites such as sugars and amino acids can act to inhibit the synthesis or activity of enzymes involved in reserve breakdown (Dunaevsky and Belozersky, 1993; Thomas and Rodriguez, 1994; Bewley and Black, 1994). Catabolite repression plays a

role in the regulation of aminopeptidases and glyoxylate cycle enzymes in cucumber (Davies and Chapman, 1980; Graham *et al.*, 1994), and of α-amylases (starch-hydrolyzing enzymes) in rice (Thomas and Rodriguez, 1994; Yu *et al.*, 1996). Metabolite accumulation may also alter the expression of reserve-mobilizing enzymes by osmotic regulation (Yu *et al.*, 1996; Thomas and Rodriguez, 1994).

The transport of reserve products from the storage tissue to the seedling axis may also be subject to control. In barley, for example, the uptake of amino acids arising in the endosperm by the scutellum may be regulated in part at the level of the transporters in the scutellum (Nyman *et al.*, 1983).

In cereals, specific gibberellins secreted by the germinating embryonic axis induce *de novo* synthesis of α-amylase in the aleurone layer of the endosperm. The α-amylase is then secreted into the endosperm, where it participates in starch breakdown (Jacobsen *et al.*, 1995). The synthesis of other enzymes required for storage reserve breakdown, such as endopeptidases, carboxypeptidases, and oligosaccharide-degrading enzymes, is also stimulated by gibberellins in most cereal species (Bewley and Black, 1994; Dominguez and Cejudo, 1995). Exogenously-supplied ABA antagonizes the effects of gibberellins in cereals (Bewley and Black, 1994).

Hormones may also act as a signal between the seedling and storage tissue in dicotyledonous species. There is circumstantial evidence that gibberellins may be supplied to the endosperm by the seedling in castor bean (Gifford *et al.*, 1984). The gibberellins appear to act as general transcriptional enhancers of genes already being expressed, including the genes encoding glyoxylate cycle enzymes (Martin *et al.*, 1984; Rodriguez *et al.*, 1987). Several studies have indicated that cytokinins, possibly originating within the

seedling axis, regulate events associated with reserve mobilization in the cotyledons in some species (Penner and Ashton, 1967a, 1967b; Allen *et al.*, 1984; Nandi *et al.*, 1995; Taneyama *et al.*, 1996). Other exogenously-applied hormones, such as ABA, may also affect reserve mobilization in some dicot species (Yomo and Varner, 1973; Marriott and Northcote, 1977; Rodriguez *et al.*, 1987).

Although these examples clearly demonstrate a role for the seedling axis in controlling reserve breakdown in the storage tissues of many species, other seed tissues may also affect storage reserve mobilization in the reserve tissue. For instance, in cucumber seeds, the testa has been shown to reduce the activity of enzymes involved in TAG and protein reserve breakdown within the cotyledons (Davies and Chapman, 1979b).

In addition to the controls exerted by the seedling axis on the metabolic pathways involved in storage reserve breakdown within the storage tissues, there may be other mechanisms involved in regulating the flux through these pathways. As an example, the role of intracellular compartmentalization in controlling reserve breakdown and utilization of the products of reserve breakdown has received little attention. Yet the highly compartmentalized nature of TAG catabolism suggests that physical separation of substrates and products from the enzymes that may act upon them could be an important factor in regulating synthesis of sugars from TAGs. Compartmentalization may conceivably be a factor in storage protein mobilization, as well.

In contrast to the abundance of literature on the control of reserve mobilization in angiosperm species, there have been few published reports on the regulation of events associated with reserve mobilization in conifer species. These reports have focused on the relationship between the megagametophyte and the developing seedling. Studies

examining β-oxidation and glyoxylate cycle enzymes have demonstrated that the seedling is able to modulate enzyme activity in the megagametophyte (Bilderback, 1974; Murray and Adams, 1980; Gifford, unpublished data). To date, no mechanisms by which interactions between the seedling and the megagametophyte affect storage reserve mobilization in conifers have been elucidated.

1.5 Metabolism of Arginine Arising from Storage Protein Breakdown

1.5.1 Potential Routes of Arginine Catabolism in Plant Tissues

Several routes of arginine metabolism have been documented in plants (Mazelis, 1980; Durzan and Steward, 1983). Arginine may be converted to agmatine, a polyamine, by arginine decarboxylase. Agmatine subsequently may be metabolized to a variety of other physiologically active polyamines (Tiburcio *et al.*, 1990). Polyamines are present in relatively high quantities during post-embryonic growth in the species that have been examined to date, suggesting that they play an important role in the processes of seedling growth and development (Matilla, 1996). Many of the reports on polyamines in conifer species have focused on somatic embryogenesis (e.g. Santanen and Simola, 1992; Minocha *et al.*, 1993; Amarasinghe and Carlson, 1994; Amarasinghe *et al.*, 1996; Laukkanen and Sarjala, 1997; Sarjala *et al.*, 1997).

Barnes (1962a) established that γ-guanidinobutyrate, a monosubstituted guanidine, was a product of U-[^{14}C]-arginine metabolism in several tissues from *Pinus* species. Radiolabelling experiments have demonstrated that γ-guanidinobutyrate was a product of U-[^{14}C]-L-arginine metabolism in white spruce buds both in late summer (Durzan, 1968a) and during spring bud burst (Durzan, 1969). Other monosubstituted guanidines arising from [^{14}C]-arginine metabolism in conifer tissues have also been reported, but their

identity was not determined (Durzan, 1968a; Durzan, 1968b; Durzan, 1969). Beyond these early studies in forest trees, the enzymology and significance of monosubstituted guanidines in plants have received little attention.

Another potential route of arginine metabolism is via deamination or transamination to α-keto-γ-guanidinovaleric acid. Free α-keto-γ-guanidinovaleric acid has been reported in white spruce shoots (Durzan and Richardson, 1966), but little is known about this compound in plants.

Finally, arginine may be converted to ornithine and urea via the enzyme arginase (L-arginine amidinohydrolase, EC 3.5.3.1). The urea is then metabolized to ammonium and carbon dioxide by urease, and the ammonium subsequently used in biosynthetic reactions, such as glutamine and asparagine synthesis (Thompson, 1980). In the catabolic pathway, ornithine is thought to be converted primarily to glutamate via glutamate-γ-semialdehyde (Mazelis, 1980).

As mentioned in Section 1.4.2.5, arginase activity has been characterized during the early seedling growth of four conifer species: Italian stone pine (Guitton, 1964), maritime pine, *Abies pectinata*, (Guitton, 1957), and Aleppo pine (Citharel and Citharel, 1975). Radiolabelling studies have been used to demonstrate the conversion of arginine to ornithine and urea in white spruce buds (Durzan, 1969). Arginase activity has also been reported in several angiosperm species, including pumpkin (*Cucurbita moshata* L.) seedling cotyledons (Splittstoesser 1969), jack bean (*Canavalia ensiformis* L.) seedling cotyledons (Downum *et al.*, 1983), soybean (*Glycine max* L.) seedling cotyledons and axes (Downum *et al.*, 1983; Matsubara and Suzuki, 1984; Kang and Cho, 1990), broad bean (*Vicia faba* L.) developing embryo and seedling cotyledons (Jones and Boulter,

1968; Kollöffel and van Dijke, 1975), pea (*Pisum sativum* L.) developing embryo and seedling cotyledons (de Ruiter and Kollöffel, 1982), grape (*Vitis vinifera* L.) leaves, fruit, seeds, and seedlings (Roubelakis and Kliewer, 1978), common bean (*Phaseolus vulgaris* L.) seedling cotyledons (Carvajal *et al.*, 1996), Jerusalem artichoke (*Helianthus tuberosus* L.) tubers (Wright *et al.*, 1981), ground nut (*Arachis hypogea* L.) seedlings (Desai, 1983), *Iris hollandica* bulbs (Boutin, 1982), and cotton (*Gossypium hirsutum*) seedlings (Dilworth and Dure, 1978). Despite these studies, surprisingly little is known about arginase-mediated arginine breakdown in either angiosperm or conifer species.

It is likely that all of the pathways mentioned above are operative to some degree during early seedling growth. However, the primary role of stored arginine in seeds is presumed to be as a source of nitrogen for the metabolic processes of early seedling growth. Arginase-mediated arginine breakdown leads to two of the four nitrogens in arginine becoming available for use in other reactions. Thus, it would be predicted that arginase catabolism would be the major route for assimilation of arginine arising from storage protein breakdown into metabolic and biosynthetic pathways.

1.5.2 Arginase-Mediated Arginine Metabolism

In mammals and other ureotelic species, the major form of arginase is found in the liver, and is a component of the urea (Krebs-Heinseleit) cycle (Fig. 1.3). The primary function of the urea cycle in these species is to generate urea for excretion of excess nitrogen (Jackson *et al.*, 1986). In plants and other organisms, these reactions are used primarily for arginine biosynthesis and catabolism (Davis, 1986; Polacco and Holland, 1993), but may also serve as a point of integration of nitrogen metabolism with the TCA cycle (Durzan and Steward, 1983). In *Saccharomyces cerevisiae* and *Neurospora crassa*,

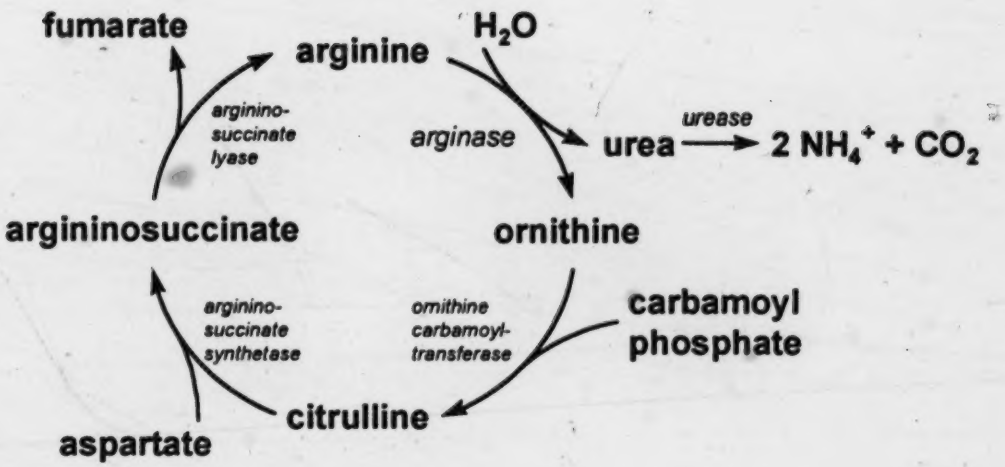

FIGURE 1.3 The urea cycle.

the biosynthetic and catabolic reactions are regulated in a manner that keeps their operation mutually exclusive (see Section 1.5.2.1), so that a complete cycle does not actually function in these organisms (Davis, 1986). Although far from conclusive, evidence from studies with various plant species, including white spruce, suggest that all four reactions of a functional urea cycle may operate in some tissues at some points in development (Durzan, 1969; de Ruiter and Kollöffel, 1982; Micallef and Shelp, 1989b).

1.5.2.1 Regulation of Arginase

The regulation of arginine catabolism via arginase in plants has received little attention. Matsubara and Suzuki (1984) demonstrated that arginase activity in developing soybean seedlings was greater in cotyledons incubated with the seedling axis than in cotyledons incubated without the axis. However, the method used to prepare the seed tissues for incubation on filter paper had a greater effect on arginase activity than whether or not the axis was attached to the cotyledon, confounding interpretation of the results. The polyamines putrescine and spermidine have been shown to increase *in vitro* arginase activity in soybean seedlings (Kang and Cho, 1990).

In lichens, there are three forms of arginase: a constitutive enzyme, an inducible enzyme, and a secreted enzyme (Legaz and Vicente, 1982; Planelles and Legaz, 1987). The inducible arginase is activated *in vivo* by L-arginine, and is inhibited by urea (Legaz and Vicente, 1980, 1982; Vicente and Legaz, 1985). Increased levels of L-arginine in the cytosol also modestly increase the levels of the constitutive arginase above baseline values (Martin-Falquina and Legaz, 1984). Different isoforms of arginase have not been demonstrated thus far in plant species.

The mechanisms involved in regulation of arginase-mediated arginine catabolism are much better understood in *Saccharomyces cerevisiae* and *Neurospora crassa*. These organisms have developed exquisite means of regulating arginine breakdown via arginase, primarily to control the balance between arginine synthesis and arginine breakdown, and are summarized in Davis (1986).

In *N. crassa*, the levels of arginase and other enzymes involved in arginine catabolism are comparably high, even when no arginine is present in the medium (Weiss and Davis, 1973). Levels of both arginase and ornithine transaminase, which synthesizes glutamate-γ-semialdehyde from ornithine in the conversion of arginine to glutamate, may be induced further by the addition of arginine to the medium (Weiss and Davis, 1977). Arginase activity can also be regulated by the availability of its cofactor, Mn^{2+}. However, most of the regulation of arginine catabolism in *N. crassa* occurs as a result of compartmentalization. Arginase is a cytosolic enzyme in *N. crassa* (Weiss and Davis, 1973). Free arginine levels in the cytosol are maintained at low levels by incorporation of arginine into proteins, and by uptake into the vacuole (Weiss, 1973). Therefore, even though ample levels of arginase are present under most conditions of growth, sequestration of arginine away from the enzyme prevents arginine catabolism. Cytosolic levels of arginine only increase when uptake of arginine from the surrounding medium by the cell exceeds the capacity for uptake of arginine into the vacuole. The arginine in the cytosol is immediately metabolized by existing arginase, producing ornithine and urea (Weiss and Davis, 1977). The *de novo* synthesized ornithine is metabolized by ornithine transaminase, along with vacuolar ornithine that is displaced by large quantities of arginine entering the vacuole. Arginine also enters the mitochondria, where it acts to inhibit two

enzymes of the ornithine biosynthetic pathway, acetylglutamate kinase and acetylglutamate synthase. By inhibiting ornithine synthesis, a futile cycle of arginine synthesis and catabolism is avoided (Davis, 1986).

In contrast to *N. crassa*, all of the enzymes of the urea cycle are cytoplasmic in *S. cerevisiae*, but are not present at high levels in the absence of arginine (Magasanik, 1992). The addition of arginine to the medium acts to greatly increase levels of arginase and ornithine transaminase in *S. cerevisiae* (Whitney and Magasanik, 1973; Magasanik, 1992) through the complex *ARG 80-82/CAR 80-82* system (Smart *et al.*, 1996). As arginine catabolism is activated, arginine biosynthesis is concomitantly diminished by inhibition of ornithine transcarbamoylase, which synthesizes citrulline from ornithine (Eisenstein *et al.*, 1986). Inhibition is achieved by the aggregation of one molecule of arginase with one molecule of ornithine transcarbamoylase. The aggregation does not affect arginase activity, while ornithine transcarbamoylase is rendered inactive (Eisenstein *et al.*, 1986).

Both *S. cerevisiae* and *N. crassa* control the rate of arginine breakdown via arginase by limiting the amount of arginine taken up by the cells in the presence of the preferred nitrogen sources ammonium and glutamine (Davis, 1986; Magasanik, 1992). This mechanism, referred to as inducer exclusion, has been well characterized in *S. cerevisiae* (e.g. Courchesne and Magasanik, 1983; Cooper *et al.*, 1992), and is thought to operate in a similar manner in *N. crassa* (Davis, 1986). The plasma membrane general amino acid permease of *S. cerevisiae* has a rapid turnover, with the amount of functional permease contingent on the balance between synthesis, inactivation, and reactivation of inactive permease molecules. In the presence of adequate quantities of the preferred substrates ammonium and glutamine, the level of functional permeases declines.

Ammonium appears to increase the rate of permease inactivation, while glutamine acts to repress the synthesis of new permease. Therefore, the amount of amino acids - including arginine - taken up from the surrounding medium is reduced, and pathways involved in their catabolism are not activated (Magasanik, 1992).

It is clear that in both *S. cerevisiae* and *N. crassa*, arginase-mediated arginine catabolism is an intricately regulated process that responds rapidly to changing nutrient conditions. Although gene expression is invoked to varying degrees as a mechanism in the regulation of arginase in both of these organisms, a significant proportion of the control of arginine flux via arginase is achieved independently of changes in gene expression.

1.6 Application of Knowledge about Early Seedling Growth to the Forest Industry

Approximately 1 billion loblolly pine seedlings are produced each year (Boyer and South, 1984). Approximately one-sixth of the seed - about 20 500 pounds - used to produce these seedlings comes from second-generation select trees (South, 1990; J. Hearnsberger, personal communication). However, the gains realized from using seed from high-value clones are reduced because of sub-optimal seed efficiency, defined here as the number of plantable seedlings in a nursery bed at time of lifting as a percentage of the number of full live seeds sown. It has been predicted that an improvement of seed efficiency from the current average of 60% to 90% would result in a 50% increase in the present net value of the seeds, a figure based on the value of the harvested wood that is ultimately produced from the planted seeds (South, 1990). The greatest mortality of conifer seedlings occurs during the first growing season, particularly during seedling establishment (Daniels and Simpson, 1990). Therefore, an understanding of the processes by which seedlings use storage reserves to sustain the earliest stages of growth and

development, and knowledge of how these biochemical pathways are controlled, may provide insight leading to improvements in nursery practices to reduce seedling mortality.

Adequate, consistent production of quality seed from top-performing clones is a constraint to seedling production. Trees do not produce reliable amounts of seed each year; rather, seed crops can be sporadic, and are influenced by weather and other environmental factors (Fowells, 1965). Somatic embryogenesis has received wide attention as a technique to achieve rapid, season-independent propagation of high-value genotypes (Gupta *et al.*, 1993; Becwar and Pullman, 1995; Litz and Gray, 1995). Somatic embryogenesis also provides a means of producing genetically altered trees by regenerating plantlets from transformed embryogenic tissue (e.g. Loopstra *et al.*, 1990; Tian *et al.*, 1997). The latter is a particularly attractive means of augmenting conventional tree breeding programmes, due to the long generation time of conifers compared to conventional crop species such as cereals (Becwar *et al.*, 1997). Loblolly pine represents a logical choice for integration of somatic embryogenesis into tree improvement programmes, not only because of its commercial value, but also because of the intensive breeding programmes and forest management practices that are already in place. Hence, many forestry and reforestation companies, both in the U.S. and elsewhere, are attempting to develop loblolly pine somatic embryogenesis protocols (Gupta *et al.*, 1993; Becwar and Pullman, 1995; Becwar *et al.*, 1997; S. M. Attree, personal communication). However, pines have not been as amenable to tissue culture as have other conifer species, so progress towards vigorous somatic embryo-derived plantlets of pines has lagged behind that of other conifers (Tautorus *et al.*, 1990; Becwar and Pullman, 1995; Klimaszewska, 1997; Becwar *et al.*, 1997). Much of the problem can be attributed to poor conversion of

somatic embryos to plantlets (Klimaszewska, 1997). One of the difficulties in producing plantlets from somatic embryos is that the embryo does not have the benefit of the megagametophyte to nourish early seedling growth. Therefore, understanding the composition of the nutrient milieu provided to the developing seedling from the megagametophyte, and the communication between the two tissues in regulating nutrient acquisition and utilization by the seedling, represents vital knowledge in the development of protocols for efficient production of vigorous seedlings via somatic embryogenesis.

1.7 The Present Study

It is apparent from the above discussion that little is known about the generation and utilization of products that arise from seed storage reserve breakdown in conifer species, despite the importance of early seedling nutrition to efficient seedling production. The overall goal of this study was to describe the fate of key amino acids resulting from the breakdown of the major storage reserves in loblolly pine during early seedling growth. This goal can be broken down into five main objectives:

The first objective was to define the role of the megagametophyte in providing the amino acid products of storage protein breakdown to the seedling during early seedling growth. To this end, the breakdown of protein reserves in the megagametophyte during dormancy-breaking, germination, and early seedling growth was followed, and the generation of free amino acid pools described.

The second objective was to ascertain the importance of arginine for early seedling growth. Two hypotheses were tested: 1. arginine is a major amino acid comprising the protein reserves of loblolly pine, and 2. arginine represents a significant component of the free amino acid pools used to support early seedling growth.

The third objective of the study was to determine if arginase played a role in the utilization of arginine during early seedling growth. Arginase activity following seed imbibition was characterized, then the hypothesis that arginase-mediated breakdown of arginine is an important route by which arginine could be assimilated into metabolic and biosynthetic pathways to support early seedling growth was tested.

The fourth objective of this study was to identify factors that may regulate the processes of storage protein mobilization and amino acid utilization. I chose to focus on the role that interactions between the megagametophyte and the seedling play in protein reserve breakdown and amino acid utilization. The effects of stratification on storage protein breakdown were examined, as well. This latter series of experiments required a basic understanding of seed dormancy in loblolly pine, which has also been addressed in this thesis.

The final objective was to purify arginase, in order to raise polyclonal anti-arginase antibodies. These antibodies will be an important tool for future studies that examine the regulation of arginase expression in more detail.

The work presented in this thesis has culminated in the development of a working model to describe the utilization of arginine as a nutrient during the early seedling growth of loblolly pine. This model has provided a thorough baseline for further investigations into the regulation of storage protein mobilization and amino acid utilization in this laboratory.

2. MATERIALS AND METHODS

2.1 Water and Chemicals

The term Milli-Q water refers to distilled, deionized water of 18 megaohm conductivity prepared with a Milli-Q 5 Bowl Plus system (Millipore, Bedford, MA).

PITC and triethylamine were purchased from Pierce (Rockford, IL). Nessler's reagent, 2-propanol, and $NiSO_4$ was purchased from Fisher (Nepean, ON). Sodium dihydrogen orthophosphate, acrylamide, NN'-methylenebisacrylamide, SDS, acetonitrile, and sodium acetate trihydrate were purchased from BDH (Toronto, ON). TEMED and ammonium persulfate were purchased from Bio-Rad (Missisauga, ON). Glycerol was purchased from Caledon (Georgetown, ON). PPD (ICN, Costa Mesa, CA) was a gift from Dr. Joseph Polacco (University of Missouri, Columbia, MO). Unless otherwise indicated, all other chemicals used for biochemical procedures were purchased from Sigma (St. Louis, MO).

2.2 Plant Material

Pinus taeda L. seeds (open pollinated clone 11-9, collected 1992) were a gift from Westvaco (Summerville, SC), and were stored at -20°C until used. Mature desiccated seeds were surface-sterilized according to the method outlined in Groome *et al.* (1991); except that 1% (v/v) sodium hypochlorite (bleach) was used rather than 30% (v/v) H_2O_2 as the sterilizing agent. Surface-sterilized seeds were stratified in the dark at 2°C between layers of Kimpak (Seedburo, Chicago, IL) for 35 days (35 DAI_2). Germination of 35 DAI_2 seeds was carried out at 30°C under constant 19 $\mu mol\ m^{-2}\ s^{-1}$ light. Under these conditions, radicle emergence occurred at four days after imbibition at 30°C (4 DAI_{30}).

In this thesis, the term embryo is used to refer to the sporophyte in mature desiccated seeds and in stratifying seeds (0 to 35 DAI_2). In all other stages of post-embryonic growth (1 to 12 DAI_{30}), the sporophyte is referred to as a seedling. Prior to radicle emergence, seedlings were staged according to both morphology and days after imbibition at 30°C. At 1 DAI_{30}, seedlings were not visibly green, and the seed coat was not open. At 2 DAI_{30}, seedlings may have shown some signs of greening, but the seed coat was not open. By 3 DAI_{30}, the seed coat had split along the suture, but the radicle had not protruded beyond the edge of the seed coat. Seedlings at 4 DAI_{30} exhibited radicles protruding beyond the edge of the seed coat, but were no longer than one mm. After 4 DAI_{30}, seedlings were staged using the radicle length criteria outlined in Mullen *et al.* (1996). Fresh material was used for all assays except in the case of short term experiments testing the effect of the megagametophyte or exogenous arginine on arginase activity, where the tissue was quick-frozen in liquid nitrogen and stored at -70°C. Tissue used for HPLC amino acid analyses was also frozen in liquid nitrogen and stored at -70°C.

In organ localization studies, whole embryos or seedlings were divided into two parts by a single transverse cut before assaying, just below the point at which the cotyledons diverge from the hypocotyl. The shoot pole segment consisted of the cotyledons and shoot (or shoot apex), while the root pole segment was made up of the hypocotyl and root (or radicle).

2.3 Tissue Culture

For *in vitro* experiments, seed parts were excised from surface sterilized seeds or seedlings under aseptic conditions, and cultured in 9 cm Petri dishes on various media, under the conditions described in Section 2.2. Tissue was cultured as isolated

megagametophytes, isolated embryos or seedlings, or as embryos or seedlings with an intact megagametophyte but with all other seed tissues removed. Isolated megagametophytes removed from mature or 35 DAI_2 seeds were cultured as individual megagametophyte halves that, unless specified, were placed with the cut side proximal to the media. Radicle emergence for embryos cultured with intact megagametophytes consistently occurred at 2 days in culture; seedlings developed at approximately the same rate as seedlings in germination trays.

Amino acid and storage protein quantification experiments. Seed parts were cultured on 3% (w/v) Bacto-Agar (Difco, Detroit, MI) with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B. The latter three media components were filter-sterilized prior to addition to the autoclaved agar.

Amino acid export experiments. Megagametophyte halves were cultured with the cut surface proximal to the surface of 0.8% (w/v) Bacto-Agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B, in quarter-sectioned Petri plates containing 7.5 mL agar per section. The agar blocks were melted prior to analysis, and treated as an extract.

PPD inhibition studies. Seedlings with intact megagametophytes were cultured under aseptic conditions on Kimpak dampened either with water or with 1.1 mM PPD, plus 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B. All solutions were filter-sterilized.

Arginase induction experiments. Seedlings were cultured under aseptic conditions on dampened Kimpak. Seedlings cultured with intact megagametophytes were cultured with 10 mM MES (pH 5.8). Seedlings cultured without megagametophytes were cultured

with one of the following solutions: 10 mM MES (pH 5.8); 10 mM MES, 100 μM $MnSO_4$, 100 μM $NiSO_4$ (pH 5.8); 100 mM sucrose, 10 mM MES, 100 μM $MnSO_4$, 100 μM $NiSO_4$ (pH 5.8); 100 mM arginine, 10 mM MES, 100 μM $MnSO_4$, 100 μM $NiSO_4$ (pH 5.8); or 100 mM arginine, 100 mM sucrose, 10 mM MES, 100 μM $MnSO_4$, 100 μM $NiSO_4$ (pH 5.8). All solutions were filter-sterilized. For seedlings cultured without megagametophytes, the Kimpak was folded over, and the seedlings placed so that the cotyledons were slightly embedded in the ridge of the Kimpak.

Exudate Collection. Intact megagametophytes were gently removed from 9 and 10 DAI_{30} seedlings, and placed in a small indentation made in an agar plate so that the corrosion cavity from which the seedling had been removed was upright (see Fig. 3.13). Megagametophytes were cultured on 3% (w/v) Bacto-Agar plates with 15 μg/mL rifampicin and 2.5 μg/mL amphotericin B. After 18 to 24 h, the exudate which had filled the corrosion cavity was removed using a 10 μL pipettor fitted with a gel loading tip. Exudate obtained from individual megagametophytes was pooled for analyses.

2.4 Protein Extractions

Protein extracts generally were prepared according to the differential extraction procedure of Gifford *et al.* (1982). Five to ten seed parts were homogenized in a mortar and pestle on ice in 1 mL 50 mM sodium phosphate buffer (pH 7.5), then microfuged at 16 000 g (14 000 rpm) for 15 min at 4°C. A portion of the supernatant was removed using a drawn-out Pasteur pipette, taking care to avoid removing any of the lipid layer on top of the supernatant, and saved for analysis. Proteins in the phosphate buffer extracts are referred to as buffer-soluble proteins. The remainder of the supernatant, along with as much of the lipid layer as possible, was removed with a Pasteur pipette. Residual lipids

along the sides of the microfuge tube were removed with a cotton swab. The pellet was washed three times in phosphate buffer as described above. A fitted pestle was used to resuspend the pellet. Proteins not soluble in phosphate buffer were solubilized by resuspending pellets in 500 µL Laemmli buffer (62.5 mM Tris·HCl [pH 6.8], 2% [w/v] SDS, and 10% [v/v] glycerol), and heating the suspensions at 95°C for 5 min, before microfuging 16 000 g (14 000 rpm) for 15 min at room temperature. A portion of the supernatant was removed with a drawn-out Pasteur pipette. Proteins in the Laemmli buffer extract are referred to as buffer-insoluble proteins. For total seed protein, tissues were extracted four times in phosphate buffer and four times in Laemmli buffer, and determined as the sum of the protein content of each of these washes. Three independent biological replicates were analyzed in duplicate for each data point.

2.4.1 Extraction of Storage Proteins for HPLC Amino Acid Analysis

To ensure removal of contaminating lipid and carbohydrate from buffer-insoluble extracts prepared for HPLC analysis of storage protein hydrolysate, the Gifford *et al.* (1982) procedure was modified to include ice-cold chloroform:methanol (2:1 v/v) and 80% (v/v) ethanol washes. Megagametophytes were extracted twice in 1 mL ice-cold chloroform:methanol (2:1 v/v), twice in 1 mL 0.05 M phosphate buffer containing 0.1 mM leupeptin, twice with ice-cold 80% ethanol, and once again with 1 mL phosphate buffer plus leupeptin. Buffer-insoluble proteins were extracted by heating the pellets at 95°C for 5 min in 500 µL Laemmli buffer. Proteins were precipitated from 50 µL aliquots of the resulting supernatant with ice-cold 80% (v/v) acetone. The pellet was rinsed twice with 80% (v/v) acetone to remove as much SDS from the sample as possible. Four independent replicates were prepared for protein hydrolysis.

2.5 Protein Assays

Protein content generally was assayed using the method of Lowry *et al.* (1951). For solutions beyond the lower detection limits of the Lowry assay, the Bradford microassay was used according to the manufacturer's directions (Bio-Rad, Missisauga, ON). BSA was used to generate standard curves for both assays. Each sample was assayed in duplicate.

2.6 *In Vivo* Protein Labelling

Ten isolated embryos or seedlings were incubated with 100µL of 100 µCi ^{35}S-met (Amersham, Oakville, ON) in unsealed Petri dishes for 3 h under the germination conditions described in Section 2.2. After labelling, the tissue was rinsed thoroughly with Milli-Q H_2O, then extracted in 500 µL phosphate buffer as described in Section 2.4. Two independent biological replicates per treatment were cultured and labelled.

Levels of ^{35}S-met uptake were determined by spotting 10 µL of extract on a Whatman GF/C filter. Incorporation of ^{35}S-met into proteins was determined by TCA precipitation. Briefly, 10 µL of sample was spotted onto a 2.4 cm GF/C filter (Whatman, Maidstone, England); the filter was washed three times with 10 mL volumes of ice-cold 5% (w/v) TCA, heated at 90°C with 1 mL of 10% (w/v) TCA, washed twice with 10 mL volumes of ice cold 10% TCA, then washed three times with 5 mL volumes of absolute ethanol. Radioactivity was determined as dpm with a Beckman LS6000TA scintillation counter (Missisauga, ON), using Amersham (Oakville, ON) ACS aqueous liquid scintillant.

2.7 Polyacrylamide Gel Electrophoresis

All electrophoresis described in Sections 2.7.1 and 2.7.2 was carried out using constant voltage with Bio-Rad (Missisauga, ON) equipment.

2.7.1 One-dimensional electrophoresis: SDS-PAGE

Protein extracts were prepared for SDS-PAGE by diluting 1:1 in Laemmli buffer with or without 5% (v/v) β-mercaptoethanol, then boiling for 5 min. Discontinuous SDS-PAGE was carried out on 0.75 mm slab gels at 200 V with the Mini-Protean II Dual Slab Cell system. A 4% T acrylamide stacking gel was used for all gels; various concentrations of acrylamide were used for the separating gel, depending on the apparent molecular masses of the proteins of interest.

For Coomassie blue or silver-stained gels, the following molecular mass standards (Sigma, St. Louis, MO) were used: phosphorylase b (97.4 kD), BSA (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). For autoradiography of radiolabelled proteins, [^{14}C]-methylated molecular mass markers (Amersham, Oakville, ON) were used: myosin (200 kD), phosphorylase b (92.5 kD), BSA (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), and lysozyme (14.3 kD).

2.7.2 Two-dimensional electrophoresis: IEF/SDS-PAGE

Protein extracts were prepared by adding the required amount of solid urea to a known volume of sample to give a final urea concentration of 9.5 M. The volume of the 9.5 M urea sample was then determined so that accurate measures of protein or radioactivity concentrations could be made.

The IEF (first) dimension was carried out with the Mini-Protean II 2-D Cell system according to the manufacturer's instructions. Tube gels (9.2 M urea, 4% T polyacrylamide [30% T/5.4% C], 2% [v/v] Triton X-100, 1.6% [v/v] Pharmalyte 5-8 ampholyte [Pharmacia, Uppsala, Sweden], 0.4% [v/v] Biolyte 3-10 ampholyte [Bio-Rad, Missisauga, ON]) were pre-electrophoresed at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min before sample application at the cathodic end of the tube. Focusing was at 500 V for 10 min, followed by 750 V for 3.5 h, using 20 mM NaOH for the cathodic (upper chamber) buffer and 10 mM H_3PO_4 for the anodic (lower chamber) buffer.

The SDS-PAGE (second) dimension was carried out as described in Section 2.7.1, except that 1 mm slab gels were used.

2.7.3 Staining and Fluorography

For general protein profile analysis, gels were stained with Coomassie Blue R. In instances where enhanced sensitivity was required, gels were silver-stained according to the manufacturer's directions (Bio-Rad, Missisauga, ON).

One- or two-dimensional gels of radiolabelled proteins were prepared for autoradiography using En^{3}Hance (Dupont, Markham, ON), then dried onto 3 mm chromatographic paper (Whatman, Maidstone, England) before exposure to X-ray film (X-OMAT AR, Kodak, Rochester, NY) at -70°C.

2.7.4 Scanning Gel Densitometry

Proteins were quantified by scanning gel densitometry of Coomassie Blue R-stained SDS-PAGE profiles at 560 nm using a Beckman (Richmond, BC) DU-65 spectrophotometer. To fully resolve the four groups of storage proteins, the megagametophyte buffer-insoluble proteins were resolved on 18% separating gels, while

15% separating gels were used to resolve the embryo and seedling buffer-insoluble proteins. Storage proteins from megagametophytes of intact seeds were quantified by scanning three individual SDS-PAGE profiles of the replicate representing the median protein concentration of the three independent replicates for each stage of development. The storage protein content of megagametophytes cultured with or without intact seedlings was quantified by scanning SDS-PAGE profiles of the three independent replicates for each data point. Storage proteins in seedlings were quantified by scanning the SDS-PAGE profiles of two independent replicates for each data point. The peak area data obtained by scanning were used to determine the percentage of protein represented by individual proteins within the protein profile. These percentages were then used to calculate the total amount of the individual protein per total volume of extract and per seed part.

2.8 Ninhydrin Soluble Amino Acid Quantification

Soluble amino acids were quantified by ninhydrin assay (Rosen, 1957). Five to ten seed parts were extracted in 50 mM sodium phosphate buffer (pH 7.5) as described in Section 2.4. Subsamples of the extract were boiled for 15 min with 50 μM sodium cyanide, 650 mM sodium acetate (pH 5.3-5.4), 0.75% (w/v) ninhydrin, and 25% (v/v) methyl cellosolve (ethylene glycol monomethyl ether) in a final volume of 2 mL. Five mL of 2-propanol was added to each tube and the samples allowed to cool at room temperature for 25 min in the dark before measuring the aborbance at 570 nm. L-alanine was used to generate the standard curve. Since different amino acids react with the ninhydrin reagent to varying degrees, the data are expressed as "L-alanine equivalents". To minimize protease activity, 0.1 mM leupeptin was included in the phosphate buffer.

The presence of leupeptin in the extract did not affect absorbance values of the ninhydrin reaction. Three independent replicates were assayed in triplicate for each data point.

2.9 Free Amino Acid Extraction for HPLC Analysis

Free amino acid extracts were prepared for HPLC analysis by the method of Tuin and Shelp (1994). Approximately 0.25 g (fresh weight) of seed parts were homogenized in 3 mL ice-cold 80% ethanol with a mortar and pestle on ice. As an internal standard, 600 nmol norleucine was added to each sample during homogenization. Samples were centrifuged at 16 000 g (14 000 rpm) for 10 min at 4°C in a Beckman J2-21 M/E high speed centrifuge and the supernatant drawn off. The pellet was re-extracted three times in 3 mL ice-cold 80% ethanol. The four supernatants were pooled, and dried under a gentle stream of filtered air. The residue was suspended in 3 mL Milli-Q H_2O; 3 mL of chloroform was added, and the mixture vortexed. The samples were then centrifuged at 1240 g (2600 rpm) for 10 min at room temperature in a clinical centrifuge to separate the aqueous and organic phases. The aqueous (top) phase was removed with a drawn-out Pasteur pipette, and filtered first through a Millex-GV 0.22 µm syringe filter (Millipore, Missisauga, ON), then through a 10 000 NMWL Ultrafree MC polysulfone membrane filter unit (Millipore, Missisauga, ON) to remove residual proteins. Fifty microlitre subsamples of filtered extract were vacuum-dried in methanol-washed 500 µL microfuge tubes using a Speed-Vac concentrator (Savant, Farmington, NY) prior to derivatization. At least three independent replicates were analyzed per data point.

2.10 Protein Hydrolysis

Acetone-precipitated storage proteins prepared as described in Section 2.4.1 were resuspended in 500 µL constant boiling 6 N HCl containing 0.1% phenol. Ten percent of

each sample was brought up to a final volume of 150 µL constant boiling 6 N HCl plus 0.1% phenol for hydrolysis. Hydrolysis was carried out in evacuated, sealed thick-walled borosilicate glass tubes for 1 h at 160°C. Cooled ampoules were opened, and samples vacuum dried in a Speed-Vac concentrator. Samples were stored at -70°C until derivatized.

Lysozyme was also analysed, and the results compared to published values, to ensure the accuracy of the technique.

2.11 HPLC Analysis

Samples were derivatized with PITC according to the Waters Manual for Pico•Tag Amino Acid Analysis (WM02, Rev. 1). The procedures in this manual are based on those outlined in Bidlingmeyer *et al.* (1984). The dried free amino acid aliquots were resuspended in 10 µL of redry solution (methanol:1 M sodium acetate:triethylamine [2:2:1 v/v/v]), then vacuum dried as described in Section 2.9. The redry step was repeated once. Samples were then derivatized by resuspending the redried residue with 20 µL of derivatizing solution (methanol:water:triethylamine:PITC [7:1:1:1 v/v/v/v]), incubating at room temperature for 10 min, then vacuum dried. Protein hydrolysates were redried twice with ethanol:water:triethylamine (2:2:1 v/v/v), and derivatized with ethanol:water:triethylamine:PITC (7:2:1:1 v/v/v/v). Derivatized samples were stored at 4°C for up to 24 h, or at -20°C in a sealed container with desiccant for no more than seven days.

Reverse-phase HPLC was conducted on a Waters system (Milford, MA) equipped with a Waters 712 Intelligent Sample Processor (WISP) and a Waters System Interface Module (SIM) linking the HPLC to a Baseline 810 workstation. A 3.9 x 300 mm Pico-

Tag free amino acid analysis column maintained at 46°C by a Waters Temperature Control Module (TCM) was used according to the procedures outlined the Waters Manual for Pico•Tag Amino Acid Analysis for physiologic amino acid analysis. Eluents 1 and 2 were prepared fresh for each run by following instructions provided by John Vukovic of Waters Canada (personal communication). To prepare Eluent 1, 9.54 g of sodium acetate trihydrate and 1000.00 g of fresh Milli-Q H_2O were added to a 1 L beaker, and stirred well. The solution was adjusted to pH 6 50 with 10% (v/v) acetic acid, and filtered through a 0.45 µm Durapore filter (Millipore, Missisauga, ON) using a large glass funnel with fritted base and stopper affixed to a glass vacuum flask. Then 975.00 g of solution was decanted gently into a 1 L beaker, 19.65 g acetonitrile and 250 µL of 10 mM EDTA were added, and the solution stirred thoroughly. The eluent was degassed before use by stirring vigorously while under vacuum for no more than 2 min. Eluent 2 was prepared by adding 353.70 g acetonitrile, 400.00 g fresh Milli-Q H_2O, 118.65 g methanol, and 250 µL of 10 mM EDTA to a 1 L beaker, mixing thoroughly, then filtering and degassing as described above. Samples were resuspended in 100 to 400 µL Waters diluent and transferred to limited volume inserts within WISP vials just prior to analysis. The standard gradient for separation of physiologic amino acids outlined in the Waters Manual for Pico•Tag Amino Acid Analysis was used for all amino acid analyses. Sigma (St. Louis, MO) acidic/neutral and basic physiologic amino acid standards and Beckman (Missisauga, ON) hydrolysate amino acid standards were used for peak identification and subsequent calculations. Selected samples were analysed in duplicate to ensure repeatability.

2.12 Arginase Activity and Endogenous Urea Determinations

Five to ten seed parts were extracted in 1 mL extraction buffer [0.1 M Tris·HCl, 0.5 mM $MnSO_4$, 0.75 mM maleate, pH 9.15) as described by Martin-Falquina and Legaz (1984). The enzyme was activated by pre-incubating extracts for 60 min at 30°C prior to assay. Enzyme activity was assayed by incubating 100 µL of the activated extract with 0.285 M arginine (pH 9.7), 1 mM $MnSO_4$, and 1 mM maleate in a total volume of 1.5 mL for 30 min at 30°C; the reaction was terminated with 0.7 vol 87% (v/v) acetic acid (Greenberg, 1955). Urea production was measured by the colorimetric method of Geyer and Dabich (1971). To account for any endogenous urea present in the crude enzyme extract that might artificially inflate the quantity of urea measured in the reaction mixture, the assay was also performed with a boiled aliquot subsampled from each extract. Endogenous urea levels, determined from the boiled controls, were subtracted from total urea levels to obtain a measure of urea evolved by arginase activity, expressed as µmol urea min^{-1}. A third aliquot of each extract was used for protein determination.

Selected samples were also assayed in the presence of the urease inhibitor PPD (50 µM) (Zonia *et al.*, 1995), to determine if any urease-mediated urea breakdown was occurring in the assay mixture (Appendix 3).

2.13 Arginase Purification

Unless indicated otherwise, all purification procedures were carried out at 4°C. PMSF (1 mM) was added to all buffers.

Approximately 90 g of shoot poles from 9-12 DAI_{30} seedlings were homogenized using a mortar and pestle in 300 mL TMG buffer (50 mM Tris·HCl [pH 7.5], 1 mM $MnCl_2$, 10% [v/v] glycerol). The tissue was further macerated with a Polytron

homogenizer (Brinkmann, Westbury, NY) at maximum speed for 20 seconds. The homogenate was centrifuged at 25 000 g (18 000 rpm) for 30 min at 4°C in a Beckman J2-21 M/E centrifuge and the resulting supernatant filtered through Miracloth (Calbiochem, La Jolla, CA). The filtrate was transferred to several 30 mL Corex tubes, and incubated for 20 min with vigorous shaking in a 60°C water bath. Following this heat treatment, the tubes were quickly immersed in an icy water bath, then centrifuged at 18 000 g (15000 rpm) for 20 min at 4°C. The supernatant was then subjected to a 30-60% ammonium sulfate fractionation using standard procedures (Englard and Seifter, 1990). The 30-60% ammonium sulfate pellet was resuspended in 11 mL TMG buffer, then 1.5 mL aliquots were desalted at room temperature using 20 mL Bio-Gel P-6DG columns (Bio-Rad, Missisauga, ON) washed with TMG buffer. Fractions containing ammonium sulphate were identified by mixing 100 µL of each fraction with 100 µL of Nessler's reagent: orange precipitate indicated the presence of NH_4^+ in the sample.

Pooled desalted fractions were applied to a 1.5 x 25 cm DE-52 (Whatman, Maidstone, England) column pre-equilibrated with 500 mM Tris·HCl (pH 7.5), then equilibrated with TMG buffer. The flow rate was adjusted to 20 mL h^{-1}, and 3 mL fractions collected until the absorbance of the fractions at 280 nm returned to baseline values. Bound proteins were eluted with a 300 mL 0-250 mM KCl gradient. Arginase eluted at approximately 75 mM KCl; active fractions, determined as outlined in Section 2.12, were pooled and concentrated using an stirred cell concentrator (Amicon, Beverley, MA) fitted with an Amicon YM-10 membrane (10 000 MWCO). The concentrated sample was then applied to a 3 x 8 cm Matrex Green dye ligand column (Amicon, Beverley, MA) equilibrated with TMG buffer and adjusted to a flow rate of 25 mL h^{-1}.

Flow-through fractions (3 mL) were collected; those containing arginase activity were pooled, and applied to a 1.5 x 25 cm arginine-linked Sepharose 4B (Pharmacia, Uppsala, Sweden) equilibrated with TMG buffer, with a flow rate of 20 mL h^{-1}. Flow-through fractions (3 mL) were collected until the A_{280} values were near zero. Bound proteins were eluted with a 300 mL 0-200 mM arginine gradient. Fractions containing peak arginase activity were collected and applied to the DE-52 column, then eluted as described above.

2.14 Electroelution

After the last step in the purification, fractions that contained high arginase activity but also contained other proteins were pooled, then concentrated approximately 10-fold for electroelution. Proteins were precipitated by the gradual addition of four volumes of ice-cold acetone to the chilled sample while stirring at 4°C. The preparation was then incubated at -20°C for 15 min before centrifuging at 15 000 g (14 000 rpm) for 20 min at 4°C in a Beckman J2-21 M/E centrifuge (Promega [Madison, WI] recommended protocol). The supernatant was discarded and the pellet allowed to dry slightly before resuspending in TM buffer (50 mM Tris·HCl [pH 7.5], 1 mM $MnCl_2$). An aliquot was removed for protein and enzyme assays. The remaining sample was divided into 250 µL aliquots, diluted 1:1 with Bio-Rad reducing sample buffer (0.06 M Tris·HCl [pH 6.8], 2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 25 % [v/v] glycerol, 0.01% [w/v] bromophenol blue), and heated at 95°C for 5 min. Prepared samples were stored at -20°C until used.

Proteins were electroeluted with SDS-PAGE using a Mini Prep Cell apparatus (Bio-Rad, Missisauga, ON). Sample volumes of 500 µL were electrophoresed on a 9 cm 10.5% T separating gel with a 2.2 cm 4.0% T stacking gel using the manufacturer's suggested protocol. The electroelution was performed at 4°C; at least 100 150-200 µL

fractions were collected following elution of the bromophenol blue. Since the proteins were denatured, fractions were examined by SDS-PAGE, and arginase subunits identified by comparing the molecular weights of the eluted proteins to that of the purified protein.

2.15 Antibody Production

The best electroelution fractions were pooled, aliquotted into 15 mL sterile screw cap tubes, lyophilized, and stored at -70°C until needed. Samples were resuspended in sterile saline solution (0.9% [w/v] sodium chloride), then diluted 1:1 in Freund's complete (initial injection) or Freund's incomplete (subsequent injections) adjuvant. All details of the immunization procedure were performed by Ms. Judy Wallace and the other staff members of Biosciences Animal Services. Two female Flemish giant x French lop rabbits were immunized subcutaneously with approximately 30 µg protein for the initial injection, and two or four booster injections of approximately 20 µg protein each were made at 28 day intervals. Blood was incubated at 37°C for 2 h, then placed at 4°C overnight to allow clotting. The clot was pelleted by centrifuging at 1240 g (2600 rpm) in a Sorvall GLC-1 swinging bucket clinical centrifuge (Markham, ON), and the serum removed. Sodium azide was added to the serum to a final concentration of 0.02% (w/v), and the serum stored at -70°C until use.

2.16 Western Blotting

Following SDS-PAGE (see Section 2.7.1), gels were equilibrated for 20 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol) at 4°C. Transfer of the proteins to nitrocellulose was carried out at 4°C using the Bio-Rad Mini Transblot apparatus, according to the manufacturer's instructions (Bio-Rad, Missisauga, ON). The transfer was performed overnight at 30 V constant voltage (Mullen and Gifford, 1995a).

Transferred proteins were first visualized by staining with Ponceau S (Sambrook *et al.*, 1989), then probed with anti-arginase antiserum according to Mullen (1995) and Kunce and Trelease (1986). Membranes were blocked in Blotto (3% [w/v] Carnation nonfat dry milk, 150 mM NaCl, 20 mM Tris·HCl [pH 7.8]) for 2 h, then incubated for 2 h with a 1:250 dilution of primary antiserum in Blotto. The membranes were then washed with four changes of Blotto over the course of 1 h, then incubated for 2 h with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad, Missisauga, ON). The membranes were again washed with four changes of Blotto over the course of 1 h prior to staining with NBT (0.30 mg/mL) and BCIP (0.15 mg/mL) in 0.1 M Tris·HCl (pH 9.5) with 0.5 mM $MgCl_2$, according to the manufacturer's instructions (Bio-Rad, Missisauga, ON). The reaction was terminated by transferring the membranes to 5% (v/v) acetic acid, followed by several washes in Milli-Q water. As a control, the immunoblotting procedure was also carried out with a 1:250 dilution of preimmune serum. No reaction was observed with the preimmune serum.

2.17 Molecular Mass Determinations

The subunit molecular mass or arginase was determined using 10% separating gel SDS-PAGE by the method of Weber and Osborn (1969), with slight modifications. Three independent replicates were used to determine the subunit molecular mass. The native molecular mass of arginase was determined by its relative mobility in TM buffer on a Superose 6 column (Pharmacia, Uppsala, Sweden) using FPLC (LKB, Uppsala, Sweden). The column was calibrated using 1 mg of each of the following molecular weight standards (Sigma, St. Louis, MO): thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), BSA (66 kD), carbonic anhydrase (29 kD), and cytochrome C (12.4

kD). The void volume of the column was determined using 10 µg lambda phage (Pharmacia), since the exclusion limit of Superose 6 is approximately 4×10^4 kD, and blue dextran, most commonly used to determine void volume, is only approximately 2×10^3 kD. Total column volume was determined with 3.3 µL acetone. The column was run at 0.4 mL/min. Two independent replicates were used to determine the native molecular mass of arginase.

2.18 Manganese Requirement

Preparations of arginase that were purified approximately 10-fold by the heat treatment, ammonium sulfate precipitation, and desalting steps described in Section 2.13 were used to conduct divalent cation requirement experiments essentially as described in Carvajal *et al.* (1996). Two 1 mL aliquots were incubated with 50 mM EDTA for 30 min at 30°C, then dialyzed overnight against several changes of TG buffer (50 mM Tris·HCl [pH 7.5], 10% [v/v] glycerol) to deplete samples of EDTA and any residual divalent cations. As a control, a third 1 mL aliquot was not treated with EDTA, but was dialyzed overnight against several changes of TMG buffer. All three aliquots were incubated for 1 h at 30°C, as per the standard method for assaying arginase activity. To one divalent cation-depleted sample, $MnCl_2$ was added to a final concentration of 2 mM. $MnCl_2$ was included in the urea-generating reaction mixture for both the control sample and for the cation-depleted sample that had had $MnCl_2$ added during the 30°C incubation, but not for the other cation-depleted sample. Subsequent steps followed the standard assay procedure.

2.19 Statistics

Unless otherwise noted, error bars represent one standard error (SE) from the mean. Data in this thesis are frequently expressed as a quotient in which both the numerator and the denominator have associated error, but are not paired samples. In these cases the standard errors were calculated using the following equation:

$$se_{y/x} = \sqrt{(\bar{y}^2/\bar{x}^2)\{(se_y)^2/\bar{y}^2 + (se_x)^2/\bar{x}^2\}}$$

This equation is simplified from one presented in Stuart and Ord (1987) by making the assumption that the correlation between the two variables x and y is 0. This assumption leads to the most conservative estimate of standard error.

Statistical significance was tested for using two value t-tests assuming unequal variance, with Excel 7.0 (Microsoft, Redmond, WA).

3. RESULTS[2]

3.1 Characteristics of Dormancy-Breaking, Germination, and Early Seedling Growth in Seeds of Loblolly Pine

3.1.1 The Role of Non-Embryonic Seed Tissues in Preventing Germination

Intact non-stratified seed did not germinate after incubation for one week in favorable conditions (Fig. 3.1). Gently cracking seed coats along the suture improved germination slightly to 10% after one week. In contrast, non-stratified embryos of loblolly pine germinated readily when non-embryonic seed tissues were removed, exhibiting 100% germination after 3 days in culture. The rate of germination for embryos cultured with intact megagametophytes was nearly the same as that for isolated embryos, but was delayed slightly if the nucellar cap sheathing the micropylar end of the megagametophyte was left intact. Germination after 7 days was reduced to approximately 45% when the seed coat was removed, but the papery layer enveloping the megagametophyte was left intact.

3.1.2 Effect of Stratification and the Megagametophyte on Embryo Germination and Seedling Development

Embryos that were excised from either mature non-stratified seeds or 35 DAI_2 stratified seeds and cultured on 3% (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B with an intact megagametophyte but with all other seed tissues removed germinated at the same rate (data not shown). Subsequent seedling

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[2] A version of this chapter has been published. King JE, Gifford DJ (1997) Plant Physiology 113: 1125-1135.

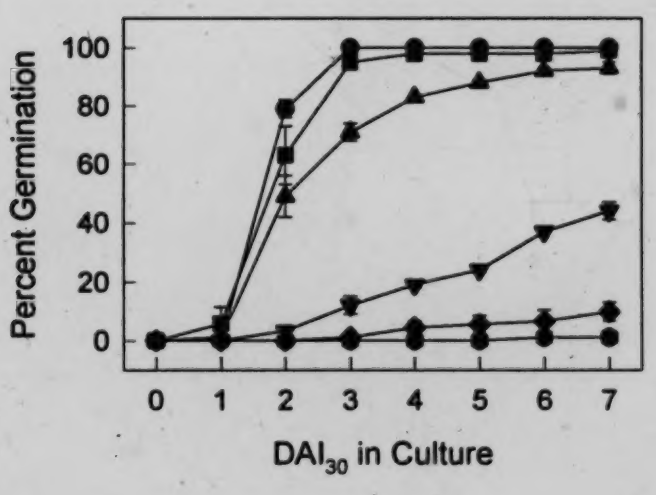
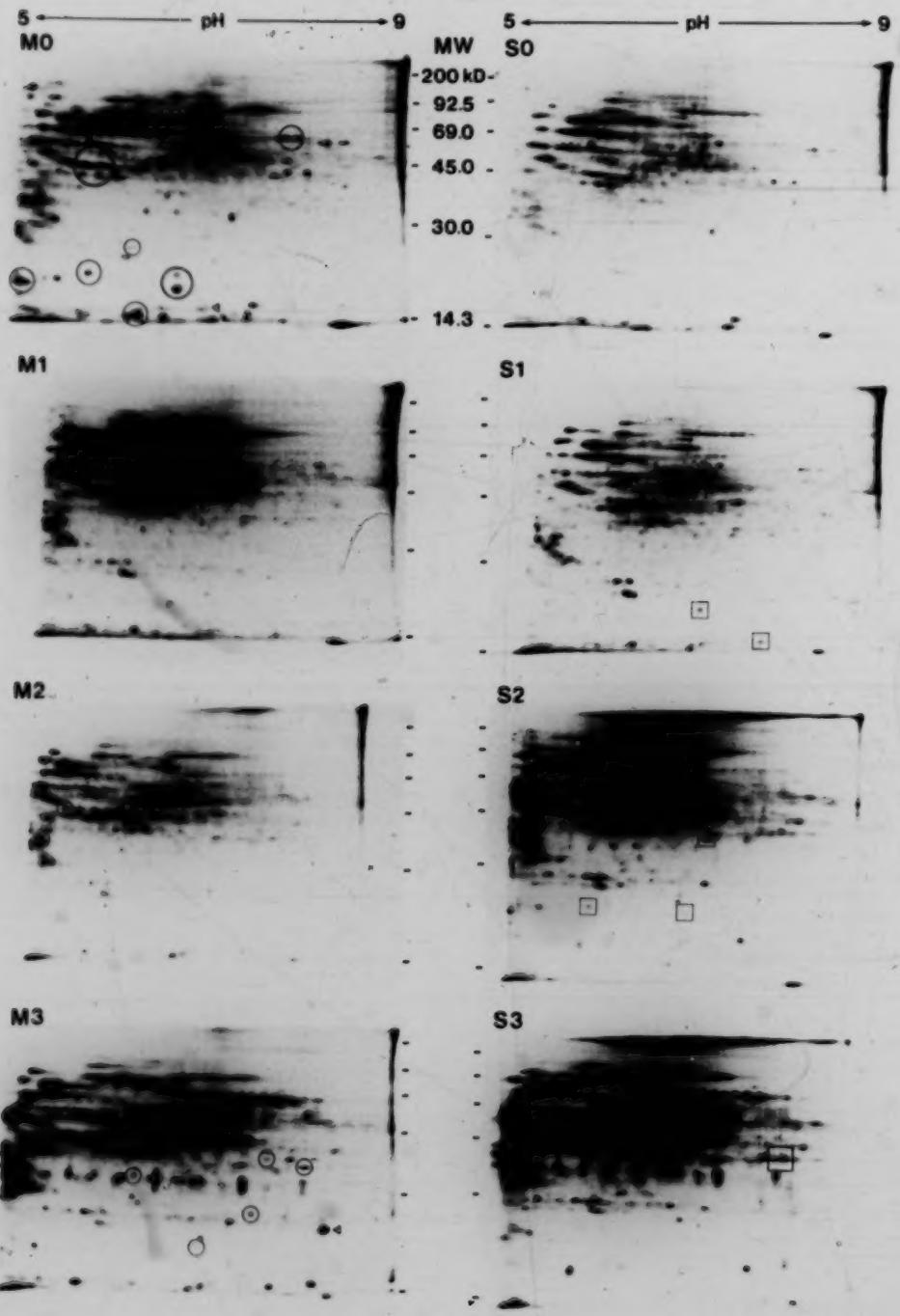


FIGURE 3.1 Effect of sequential tissue removal on germination of mature, non-stratified seeds. Isolated embryos (●); embryos with megagametophyte intact, and all other seed layers removed (■); embryos with megagametophyte and nucellar cap intact, and all other seed layers removed (▲); embryos with megagametophyte, nucellar cap, and papery layer intact, and all other seed layers removed (▼); all seed layers present, but the seed coat cracked along the suture (◆); intact seeds (●). Germination was considered to be complete when the radicle had protruded beyond the edge of the enclosing tissues; in the case of isolated embryos, completion of germination was defined as when the embryo had elongated and begun to green. Values are a mean ± SE of three independent replicates. Each replicate consisted of thirty individual seeds.

development from non-stratified or stratified embryos cultured with intact megagametophytes was practically indistinguishable. Similarly, isolated embryos excised from mature, non-stratified seeds germinated at the same rate as isolated embryos excised from 35 DAI_2 stratified seeds (data not shown). On 3% (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B, seedling development from isolated embryos was comparable to that of embryos cultured with intact megagametophytes for the first three to four days of culture. During subsequent days in culture, seedling development in the absence of the megagametophyte was visibly retarded compared to the development of seedlings cultured with megagametophytes.

In vivo protein labelling of seedlings excised from mature non-stratified seeds or 35 DAI_2 stratified seedlings and grown in the absence of megagametophytes on 3% (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B, for one, two, or three days revealed only minor differences in the proteins being synthesized by seedlings grown from mature non-stratified embryos and those synthesized by seedlings grown from isolated 35 DAI_2 stratified embryos (Fig 3.2). Stratification of the seeds prior to embryo culture did not affect the ability of the isolated seedlings to break down protein reserves: a similar pattern of storage protein breakdown occurred in seedlings cultured from either isolated mature non-stratified embryos or isolated 35 DAI_2 stratified embryos (Fig. 3.3). Storage protein breakdown during loblolly pine post-embryonic development is discussed in further detail in Section 3.2.

FIGURE 3.2 Effect of stratification on *in vivo* protein synthesis by seedlings cultured from isolated embryos. Embryos excised from mature, non-stratified seeds (M) or 35 DAI_2 stratified seeds (S) were cultured without megagametophytes on 3% (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B, for one (M1, S1), two (M2, S2), or three days (M3, S3). Seedlings were then radiolabelled *in vivo* with [^{35}S]-met. Freshly excised mature non-stratified and 35 DAI_2 stratified embryos were also labelled (M0, S0). Proteins were separated by IEF/SDS-PAGE, and [^{35}S]-met-labelled proteins visualized via fluorography. Symbols are used to indicate differences between *in vivo* protein synthesis by isolated embryos or seedlings from mature non-stratified seeds and *in vivo* protein synthesis by isolated embryos or seedlings from 35 DAI_2 stratified seeds at the same stage of development. Proteins synthesized in higher quantities by mature embryos or seedlings than by stratified embryos or seedlings (○). Proteins synthesized in higher quantities by stratified embryos or seedlings than by mature embryos or seedlings (□). Arrowheads denote proteins present in mature embryos or seedlings that were not detected in stratified embryos or seedlings.


5 pH 9
M0
MW
200 kD
92.5
69.0
45.0
30.0
14.3
5 pH 9
S0
M1
S1
M2
S2
M3
S3

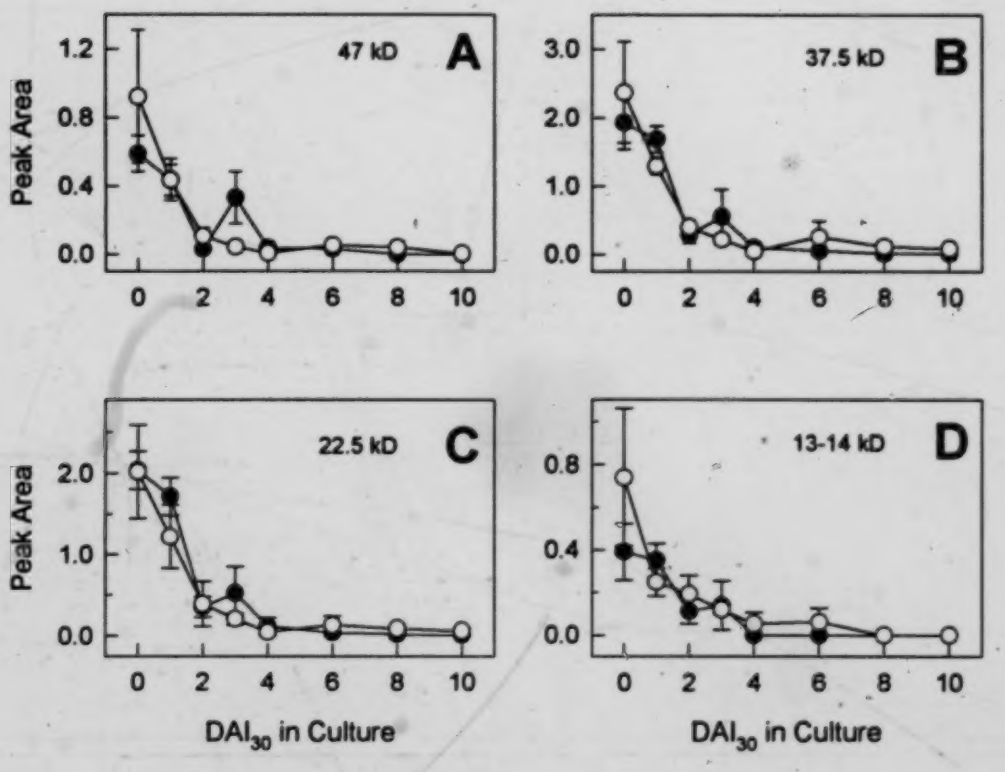


FIGURE 3.3 Effect of stratification on storage protein breakdown in seedlings cultured without megagametophytes. Isolated embryos excised from mature non-stratified seeds (●) or 35 DAI_2 stratified seeds (O) were cultured for up to 10 days on 3 % (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B. Individual storage proteins were quantified by scanning gel densitometry of 15% separating gel SDS-PAGE profiles of phosphate buffer-insoluble protein extracts from two independent replicates. Values represent the mean ± SE of the two determinations. **A**, 47-kD protein; **B**, 37.5-kD protein; **C**, 22.5-kD protein; **D**, 13- to 14-kD protein.

3.2 Storage Protein Breakdown

3.2.1 The Major Seed Storage Proteins

Groome (1991) previously demonstrated that the majority of the seed storage proteins in loblolly pine are not soluble in 50 mM phosphate buffer (pH 7.5). The major buffer-insoluble storage proteins in the mature seed megagametophyte exhibited molecular masses of 47 kD, 37.5 kD, 22.5 kD, and 13 to 14 kD (Fig. 3.4, Lane A). The 13- to 14-kD storage protein could be fully resolved using a 15% or 18% separating gel (results not shown). Seed storage proteins with the same or similar molecular masses were also identified in the embryo of mature seed (Fig. 3.4, Lane B). A group of proteins in the embryo buffer-insoluble protein profile with molecular masses ranging from approximately 15.5 to 20 kD may represent storage proteins, since they, too, disappear during post-embryonic development. Proteins with similar molecular masses were also present in the megagametophyte buffer-insoluble protein SDS-PAGE profile, but were present in lower proportions. The 15.5- to 20-kD proteins were not considered in this study.

Each of the four storage proteins identified in Figure 3.4 is actually composed of more than one isoform (data not shown). Thus, it should be noted that the terms 47-kD protein, 37.5-kD protein, 22.5-kD protein, and 13- to 14-kD protein used to refer to these proteins in this thesis actually denote four groups of closely-related polypeptides.

Peak areas obtained by scanning densitometry of 18% separating gel SDS-PAGE protein profiles of the replicate from each developmental stage representing the median protein concentration indicated that the 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD proteins made up approximately 4.5 ± 0.3%, 35.8 ± 1.2%, 26.3 ± 1.2%, and 30.5 ± 1.5% (mean ± SE), respectively, of the buffer-insoluble proteins in the mature, non-stratified

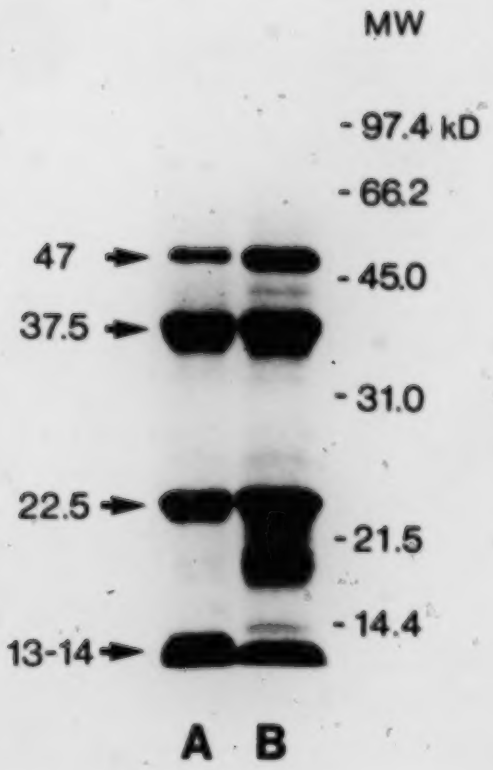


FIGURE 3.4 Seed storage proteins of loblolly pine. Coomassie blue-stained 12% SDS-PAGE profiles of buffer-insoluble proteins from the megagametophyte (lane A) and the embryo (lane B) of mature seed, in the presence of β-mercaptoethanol. The 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD storage proteins are indicated to the left of the gel. Molecular mass markers are indicated to the right of the gel.

TABLE 3.1 The Major Seed Storage Proteins in the Mature Seed of Loblolly Pine.

Seed Part	Total Buffer-Insoluble Protein[a] (µg seed part^{-1})	Storage Protein Quantification[b] (µg seed part^{-1})					Percent of Total Seed Protein
		47 kD	37.5 kD	22.5 kD	13-14 kD	Σ	
Megagametophyte	1460 ± 84	66 ± 5	522 ± 18	384 ± 17	445 ± 23	1416 ± 10	69.9 ± 2.7
Embryo	60.2 ± 4.9	6.3 ± 0.5	20.2 ± 1.7	19.8 ± 3.5	4.2 ± 0.5	50.4 ± 4.4	2.5 ± 0.2

[a] Total buffer-insoluble protein values were determined using the Lowry method, and represent the mean ± SE of three independent replicates for the megagametophyte determinations, and four independent replicates for the embryo determinations.

[b] The 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD proteins were quantified by scanning gel densitometry of Coomassie blue-stained SDS-PAGE profiles of the buffer-insoluble protein fractions. The megagametophyte storage proteins were quantified by scanning triplicate 18% separating gel SDS-PAGE profiles of the replicate representing the median protein concentration. The storage proteins in the embryo were quantified by scanning 15% separating gel SDS-PAGE profiles of two independent replicates. The peak areas obtained from the scanning gel densitometry were then used to calculate the amount of each protein present in each of the buffer-insoluble extracts from the independent replicates. See Section 2.7.4 for more details.

megagametophyte (Table 3.1). Together, these four proteins represented 97.1 ± 5.6% of the total buffer-insoluble fraction. Phosphate buffer-insoluble proteins of the megagametophyte made up 80.3 ± 1.2% of the total protein in the non-stratified megagametophyte, and 72 ± 2% of the total seed protein in the mature non-stratified seed of loblolly pine. Therefore, the four major buffer-insoluble proteins in the megagametophyte made up approximately 70% of the total protein in the mature loblolly pine seed.

In the mature non-stratified embryo, the 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD storage proteins made up approximately 10.4 ± 0.2%, 33.5 ± 0.7%, 32.9 ± 2.6% and 6.9 ± 0.5% (mean ± SE), respectively, of the phosphate buffer-insoluble proteins, for a total of 83.8 ± 2.8% of the buffer-insoluble protein in the mature, non-stratified embryo (Table 3.1). Phosphate buffer-insoluble proteins in the mature embryo made up 30.5 ± 2.5% of the total protein in the embryo, and 3.2 ± 0.2% of the total seed protein. From these data, the 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD storage proteins in the embryo were calculated to constitute about 2.5% of the total protein in the mature seed.

When expressed on a per mg dry weight basis, the megagametophyte from the mature, non-stratified seed of loblolly pine contained 4.4-fold more of the major buffer-insoluble storage proteins per unit mass (146.0 ± 1.6 μg mg^{-1} dry weight) than the embryo (33.0 ± μg mg^{-1} dry weight).

3.2.2 Storage Protein Breakdown in the Megagametophyte

The course of breakdown of the 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD storage proteins in the megagametophyte of intact seed was determined, since proteolysis

of these proteins would contribute substantially to free amino acid pools accumulated during post-embryonic development. The total protein content of the seed remained relatively constant throughout the stages of development examined (data not shown), so that quantifying individual storage proteins either as µg storage protein (data not shown) or as a percentage of total seed protein (Fig. 3.5) produced similar plots. Little proteolysis of the megagametophyte buffer-insoluble storage proteins occurred over the course of stratification and germination. Breakdown of these proteins mainly occurred following radicle emergence (4 DAI_{30}). The 37.5-kD, 22.5-kD, and 13- to 14-kD storage proteins shared a similar pattern of disappearance. By 12 DAI_{30}, approximately 96 ± 5%, 86 ± 6% and 99 ± 7% of the 37.5-kD, 22.5-kD, and 13- to 14-kD proteins, respectively, had been broken down. The 47-kD storage protein, a much smaller contributor to the protein reserves than the 37.5-, 22.5-, and 13- to 14-kD proteins, was broken down more quickly following radicle emergence than the other buffer-insoluble storage proteins. Levels of this protein had declined to below detectable limits by 9 DAI_{30}.

3.2.3 Factors Affecting Storage Protein Breakdown in the Megagametophyte

3.2.3.1 Stratification

To assess the effect of stratification on the ability of the megagametophyte to elicit storage protein breakdown, seed tissues external to the megagametophyte were removed from mature non-stratified and 35 DAI_2 seeds to allow seedling development to occur at comparable rates. Proteolysis of the four buffer-insoluble storage proteins was nearly identical in non-stratified and 35 DAI_2 intact megagametophytes with enclosed seedlings over the course of 10 days in culture (Fig. 3.6 A-D).

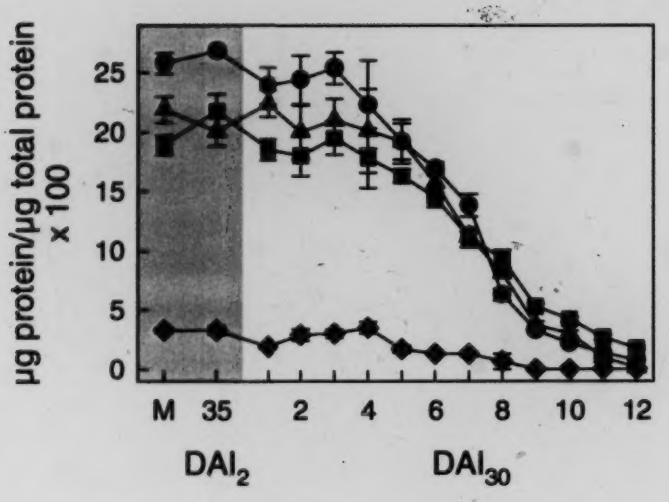


FIGURE 3.5 Quantitative changes in the 47-kD (◆), 37.5-kD (●), 22.5-kD (■), and 13- to 14-kD (▲) buffer-insoluble storage proteins in the megagametophyte of intact seeds during post-embryonic development. The data are expressed as the percentage that each of the storage proteins represents of the total protein content of the megagametophyte plus embryo or seedling at each stage of development. Total protein values are a mean of three independent replicates, analyzed in duplicate. Storage proteins were quantified by scanning gel densitometry of triplicate Coomassie blue-stained 18% separating gel SDS-PAGE profiles as described in Table 3.1. M, mature desiccated seed.

Stratification also had little effect on storage protein breakdown in isolated megagametophytes halves (Fig. 3.6 E-H), although during the latter stages of culture, there appeared to be slightly more breakdown of each of the buffer-insoluble storage proteins in isolated megagametophyte halves cultured from 35 DAI_2 seeds than in those cultured from non-stratified seeds.

3.2.3.2 Seedling-Megagametophyte Interactions

The patterns of storage protein disappearance in megagametophytes excised from mature non-stratified seed and cultured either with or without seedlings were similar (Fig. 3.7 A-D). However, the breakdown of storage proteins in isolated megagametophyte halves appeared to slow in the latter stages of culture compared to the breakdown of these proteins in megagametophytes cultured with intact seedlings. By 10 days in culture, 42 ± 14%, 45 ± 11%, 34 ± 20%, and 57 ± 22% (mean ± SE) of the 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD storage proteins, respectively, had been broken down in isolated megagametophytes, compared with 92 ± 30%, 83 ± 10%, 75 ± 8%, and 87 ± 22% (mean ± SE) breakdown of these storage proteins in megagametophytes with seedlings.

Storage protein breakdown in megagametophytes cultured with or without seedlings from 35 DAI_2 seeds was also similar (Fig. 3.7 E-H). Again, storage protein breakdown in megagametophytes without seedlings appeared to decline compared to breakdown in megagametophytes with seedlings. By 10 days in culture, isolated megagametophytes had broken down 65 ± 8%, 61 ± 9%, 57 ± 9% and 76 ± 8% (mean ± SE) of the 47-kD, 37.5-kD, 22.5-kD, and 13-to 14-kD proteins, respectively, whereas megagametophytes cultured with an enclosed seedling showed 100 ± 10%, 92 ± 10%, 82 ± 10%, and 91 ± 9% (mean ± SE) breakdown of these proteins.

FIGURE 3.6 Effect of stratification on storage protein breakdown in megagametophytes cultured with (panels A-D) or without (panels E-H) intact seedlings. Megagametophytes from mature non-stratified seeds or 35 DAI_2 stratified seeds were cultured for up to 10 days on 3 % (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B, either as intact megagametophytes with enclosed seedling, or as isolated megagametophyte halves cut along a median longitudinal sagittal plane and placed with the cut surface proximal to the surface of the agar. Individual storage proteins were quantified by scanning densitometry of 18% separating gel SDS-PAGE buffer-insoluble protein profiles of three independent replicates. Each data point represents the mean ± SE of the three replicates. Mature megagametophytes with seedlings (■); 35 DAI_2 megagametophytes with seedlings (◆); mature megagametophytes without seedlings (□); 35 DAI_2 megagametophytes without seedlings (◇). **A**, Megagametophytes with seedlings, 47-kD protein; **B**, Megagametophytes with seedlings, 37.5-kD protein; **C**, Megagametophytes with seedlings, 22.5-kD protein; **D**, Megagametophytes with seedlings, 13-14 kD protein; **E**, Megagametophytes without seedlings, 47-kD protein; **F**, Megagametophytes without seedlings, 37.5-kD protein; **G**, Megagametophytes without seedlings, 22.5-kD protein **H**, Megagametophytes without seedlings, 13- to 14-kD protein.

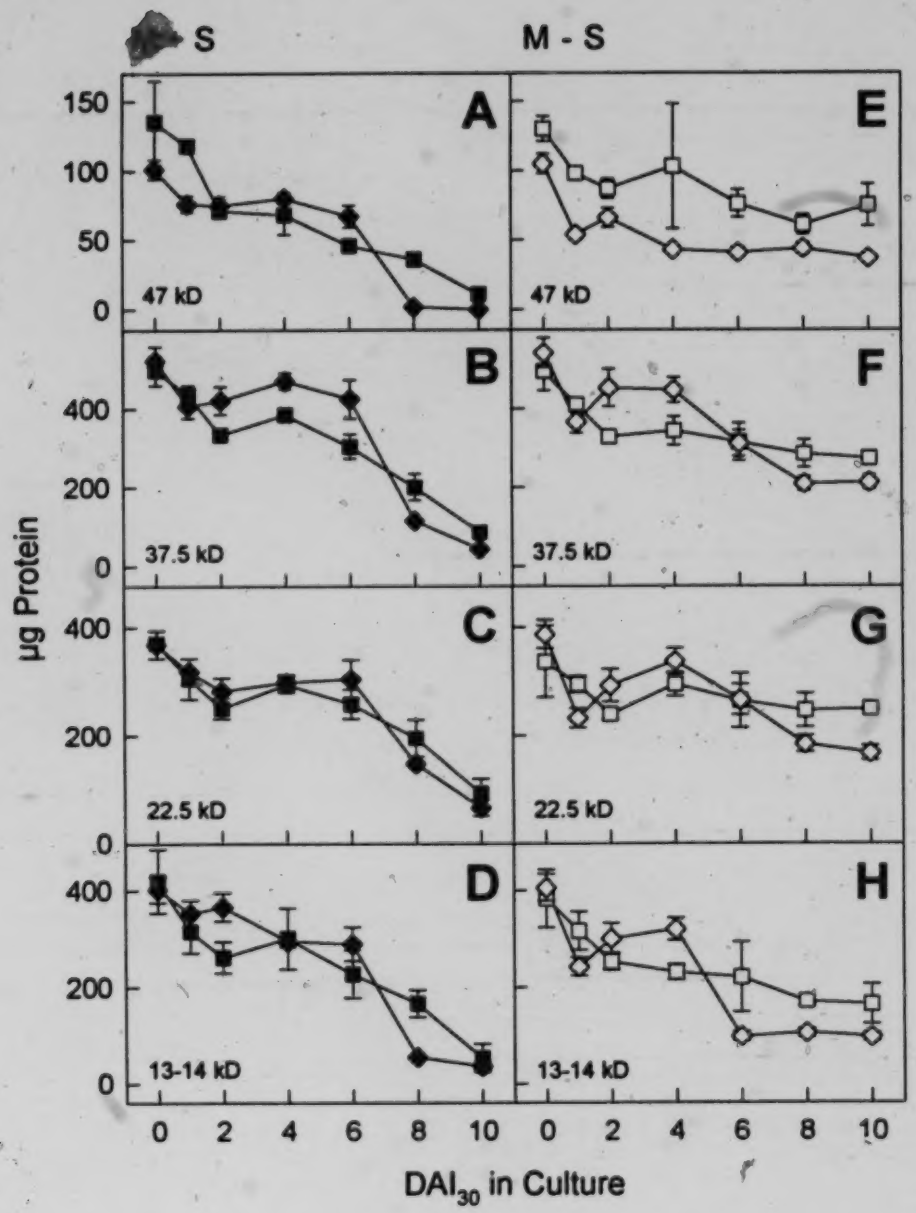
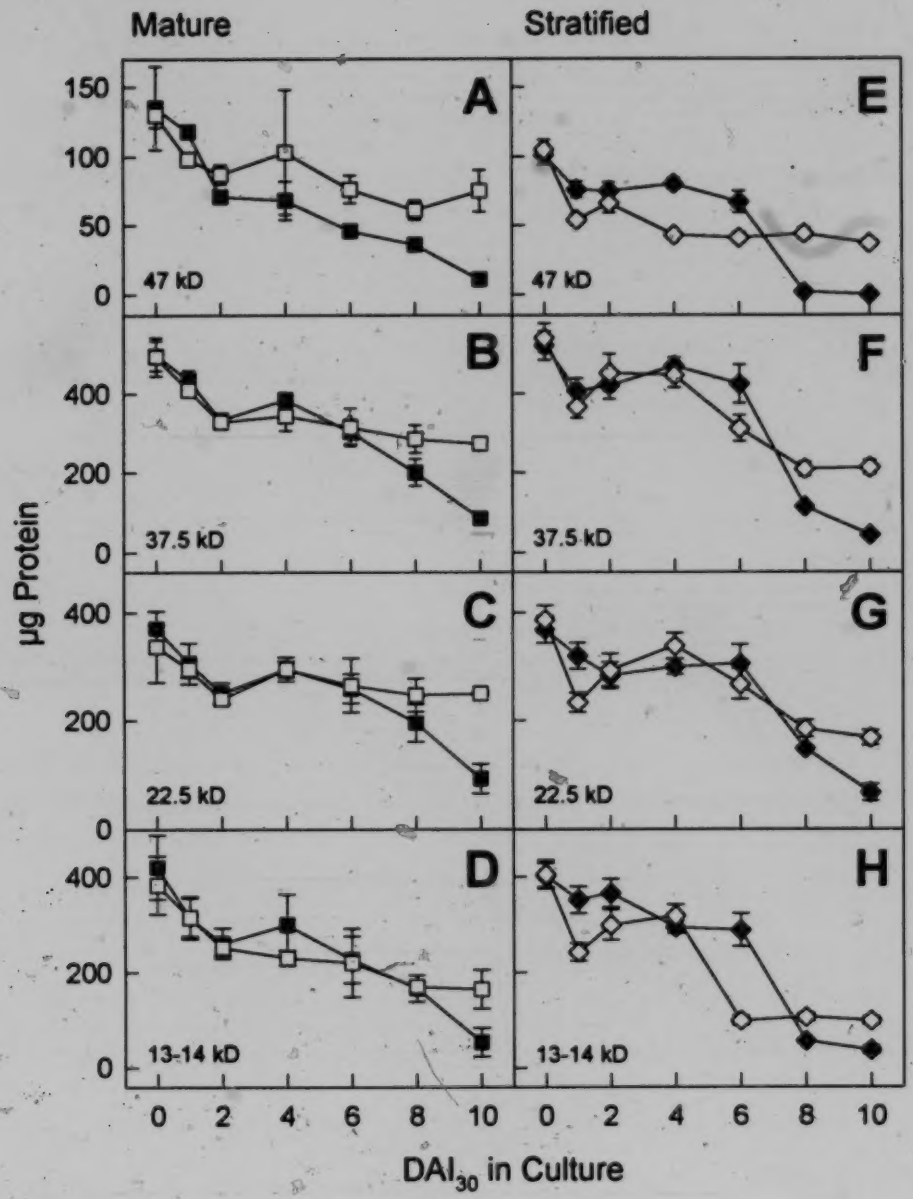

S
M - S
A
B
C
D
E
F
G
H
47 kD
37.5 kD
22.5 kD
13-14 kD
µg Protein
DAI30 in Culture
0
50
100
150
200
400
2
4
6
8
10

FIGURE 3.7 Effect of the seedling on storage protein breakdown in megagametophytes cultured from mature, non-stratified seeds (panels A-D) or 35 DAI_2 stratified seeds (panels E-H). Megagametophytes were cultured for up to 10 days on 3 % (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B, either as intact megagametophytes with enclosed seedling, or as isolated megagametophyte halves cut along a median longitudinal sagittal plane and placed with the cut surface proximal to the surface of the agar. Individual storage proteins were quantified by scanning densitometry of 18% separating gel SDS-PAGE buffer-insoluble protein profiles of three independent replicates. Each data point represents the mean ± SE of the three replicates. Mature megagametophytes with seedlings (■); mature megagametophytes without seedlings (□); 35 DAI_2 megagametophytes with seedlings (◆); 35 DAI_2 megagametophytes without seedlings (◇). **A**, Mature seeds, 47-kD protein; **B**, Mature seeds, 37.5-kD protein; **C**, Mature seeds, 22.5-kD protein; **D**, Mature seeds, 13- to 14-kD protein; **E**, 35 DAI_2 seeds, 47-kD protein; **F**, 35 DAI_2 seeds, 37.5-kD protein; **G**, 35 DAI_2 seeds, 22.5-kD protein **H**, 35 DAI_2 seeds, 13- to 14-kD protein.


Mature
Stratified
A
B
C
D
E
F
G
H
47 kD
37.5 kD
22.5 kD
13-14 kD
µg Protein
150
100
50
0
400
200
0
0 2 4 6 8 10
DAI30 in Culture

3.2.3.3 Exogenous Arginine

Levels of storage proteins, estimated by quantifying buffer-insoluble protein, were greater in isolated megagametophyte halves cultured for four or eight days with 100 mM arginine than levels in isolated megagametophyte halves cultured without arginine (Fig. 3.8). However, these difference were not significant at α=0.05, with n=2.

3.2.4 Amino Acid Composition of the Megagametophyte Storage Proteins

HPLC was used to determine the amino acid composition of acid-hydrolyzed buffer-insoluble protein extracted from 35 DAI_2 megagametophytes. The 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD storage proteins made up more than 95% of the total protein content in each of the three replicates that were analyzed. The chloroform:methanol and ethanol pretreatments used to remove contaminating lipids and carbohydrates from the buffer-insoluble proteins did not affect protein yield or SDS-PAGE profile (data not shown) when compared to the standard method for the extraction of buffer-insoluble proteins (Gifford *et al.*, 1982).

Arginine comprised 23.4 mol% of the amino acid content of the buffer-insoluble protein hydrolysate (Table 3.2). Together, glutamate and glutamine (glx) made up 21.0 mol% of the amino acid hydrolysate. Glutamine could not be distinguished from glutamate because of the acid hydrolysis procedure used for the analysis. If the most conservative assumption is made that glx is composed entirely of glutamine, yielding two N per molecule of glx, then glx constitutes approximately 20.5 ± 1.2% of the total nitrogen in the buffer-insoluble storage proteins of the megagametophyte. Arginine, with four N per molecule, contributes 45.8 ± 0.8% of the nitrogen in these proteins (Table 3.2).

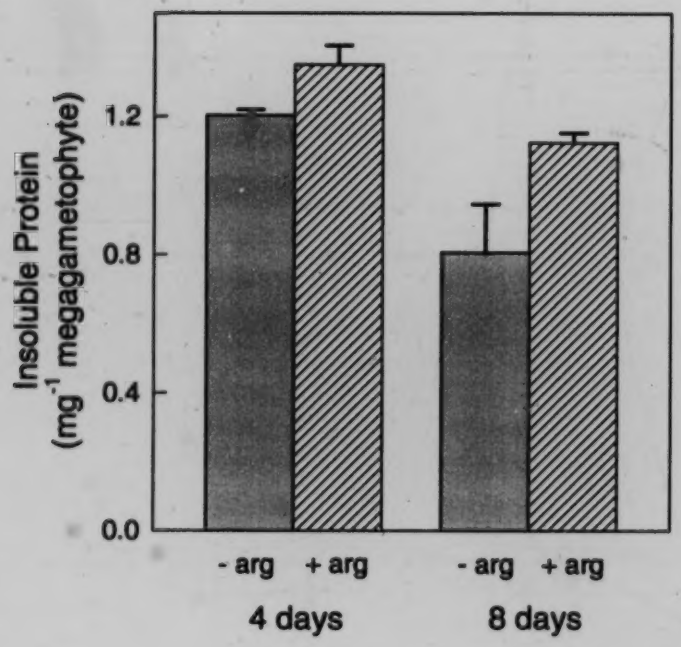


FIGURE 3.8 Effect of exogenously-applied arginine on storage protein breakdown in the megagametophyte. Isolated megagametophyte halves removed from 35 DAI_2 seeds were cultured for four or eight days on Kimpak wetted with 5 mM MES (pH 5.8) with or without 100 mM arginine. Values are a mean ± SE of two determinations, analyzed in duplicate.

TABLE 3.2 Amino acid composition of the buffer-insoluble proteins extracted from 35 DAI_2 megagametophytes.

Values are the mean ± SE of three independent replicates. Proteins were hydrolyzed with 6 N constant boiling HCl with 0.1 % phenol for 1 hr at 160°C. Glutamate and glutamine were analyzed together as glx; similarly, aspartate and aspartic acid were analyzed as asx. Cysteine and tryptophan levels were not determined.

Amino Acid	mol %[a]	mol % N[b]
Asx	6.88 ± 0.23	6.72 ± 0.22
Glx	21.00 ± 1.25	20.51 ± 1.21
Ser	6.15 ± 0.07	3.01 ± 0.03
Gly	5.81 ± 0.21	2.83 ± 0.10
His	1.69 ± 0.01	2.48 ± 0.01
Thr	1.76 ± 0.06	0.86 ± 0.03
Ala	5.28 ± 0.10	2.58 ± 0.05
Arg	23.44 ± 0.42	45.80 ± 0.84
Pro	5.15 ± 0.10	2.57 ± 0.08
Tyr	3.15 ± 0.02	1.54 ± 0.01
Val	4.14 ± 0.17	2.02 ± 0.09
Met	1.69 ± 0.09	0.83 ± 0.05
Ile	2.25 ± 0.01	1.10 ± 0.01
Leu	5.19 ± 0.07	2.53 ± 0.03
Phe	1.36 ± 0.33	0.67 ± 0.16
Lys	1.05 ± 0.05	1.03 ± 0.04

[a] Calculations based on the prediction that cys and trp together account for no greater than 4 mol % of the amino acid composition.

[b] Based on the most conservative assumption that glx = 100% gln, and asx = 100% asn.

3.3 Free Amino Acid Pools

3.3.1 Free Amino Acid Pool Quantification

Quantification of soluble amino acid levels during post-embryonic development of intact seed by ninhydrin assay demonstrated that the levels of soluble amino acids in the embryo of the mature seed (0.068 ± 0.008 µmol L-alanine equivalents per embryo) were approximately half that found in the megagametophyte from mature seed (0.184 ± 0.008 µmol L-alanine equivalents per megagametophyte) (Fig. 3.9 A). By radicle emergence (4 DAI_{30}), levels of soluble amino acids had risen to 0.230 ± 0.018 µmol L-alanine equivalents per seedling, and to 0.453 ± 0.014 µmol L-alanine equivalents per megagametophyte. By 12 DAI_{30}, levels of soluble amino acids in the seedling had increased to 10.3 ± 0.2 µmol L-alanine equivalents per seedling, whereas levels of soluble amino acids in the megagametophyte had increased to only 1.42 ± 0.22 µmol L-alanine equivalents per megagametophyte. The soluble amino acid data demonstrated the same relative changes when expressed on a per mg dry weight basis (data not shown).

Soluble amino acid pools in the root pole of the seedling from intact seed were greater than those of the shoot pole at all stages of development (Fig. 3.9 B). In 35 DAI_2 embryos, the root pole contained 0.071 ± 0.002 µmol L-alanine equivalents per root pole, while the shoot pole contained 0.046 ± 0.002 µmol L-alanine equivalents per shoot pole. By 6 DAI_{30}, the soluble amino acid pools in the root pole (0.600 ± 0.025 µmol L-alanine equivalents per root pole) were nearly 3 times greater than those in the shoot pole (0.220 ± 0.009 µmol L-alanine equivalents per shoot pole). After 6 DAI_{30}, the shoot pole accumulated proportionately greater amounts of soluble amino acids than the root pole, so that by 12 DAI_{30}, the pool sizes in the shoot pole (5.47 ± 0.31 µmol L-alanine equivalents

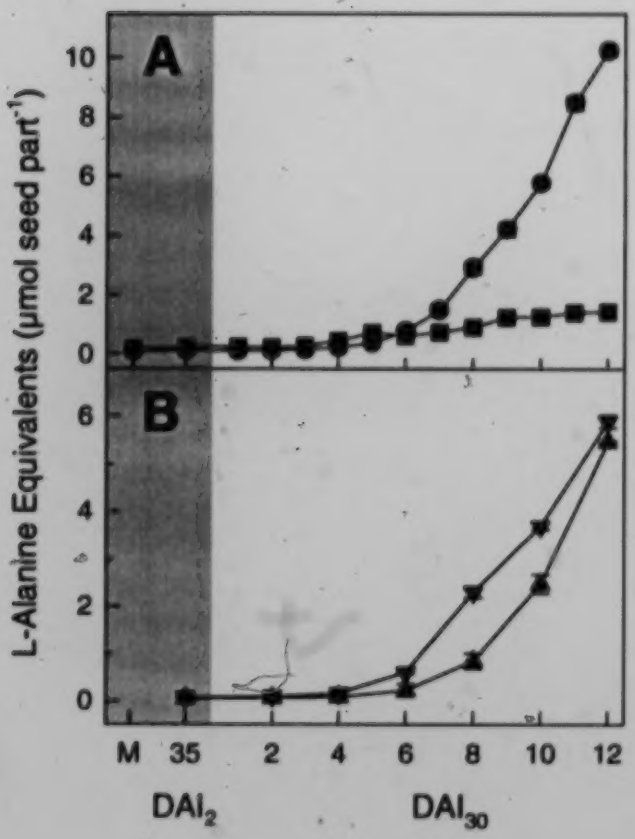


FIGURE 3.9 Quantitative changes in soluble amino acid content during post-embryonic development, determined by ninhydrin assay. **A**, Seedlings (●) and megagametophytes (■) from intact seeds. **B**, Shoot poles (▲) and root poles (▼) dissected from seedlings of intact seeds prior to assaying. Values are a mean ± SE of three independent replicates, analyzed in triplicate. M, mature desiccated seed.

per shoot pole) were relatively equal to those in the root pole (5.89 ± 0.13 µmol L-alanine equivalents per root pole).

3.3.2 Free Amino Acid Pool Composition

Relatively minor changes in the proportions of amino acids or the overall free amino acid pool size occurred during stratification, as demonstrated by a comparison of the amino acid profiles of mature non-stratified and 35 DAI_2 embryos (Table 3.3) and megagametophytes (Table 3.4). Aspartate, glutamate, asparagine, arginine, and proline were the major amino acids in the mature non-stratified and 35 DAI_2 embryo; aspartate, glutamate, asparagine, and arginine were major amino acids in the mature non-stratified and 35 DAI_2 megagametophyte. Of these amino acids, levels of aspartate and glutamate demonstrated the largest relative increases in the embryo during stratification, while glutamate showed the greatest relative increase in the megagametophyte during stratification.

Glutamate, glutamine, aspartate, and arginine were the most prominent amino acids in 3 DAI_{30} seedlings. Glutamate, glutamine, alanine, and methionine demonstrated the largest percent increases in the seedling from 35 DAI_2 to 3 DAI_{30}, although methionine was present in very low quantities. Aspartate, glutamate, and arginine were the major amino acids in the megagametophyte at 3 DAI_{30}. Serine, glutamine, alanine, and proline exhibited the greatest percent increases in the megagametophyte from 35 DAI_2 to 3 DAI_{30}.

The most pronounced changes in the amino acid profiles in the seed occurred following radicle emergence, i.e. in 6 DAI_{30}, 9 DAI_{30}, and 12 DAI_{30} seedlings. Large increases in the amounts of a few amino acids, rather than uniform increases in many amino acids, accounted for much of the rise in the amino acid content of the seedling

TABLE 3.3 Composition of the soluble amino acid pools in embryos and seedlings during post-embryonic development.

Amino acid values are expressed as nmol seedling^{-1}. Total protein is expressed as µg seedling^{-1}. Each value is the mean ± SE of three independent replicates.

Amino Acid	Mature	35 DAI_2	3 DAI_{30}	6 DAI_{30}	9 DAI_{30}	12 DAI_{30}
Asp	2.46 ± 0.35	4.82 ± 0.72	9.17 ± 1.86	6.74 ± 0.24	30.3 ± 1.4	80.1 ± 10.3
Glu	5.67 ± 0.51	10.6 ± 1.6	28.5 ± 5.6	50.5 ± 8.7	138 ± 15	248 ± 23
Ser	0.74 ± 0.02	0.77 ± 0.13	2.00 ± 0.16	13.7 ± 2.3	53.2 ± 4.2	152 ± 2
Asn	2.88 ± 0.15	3.05 ± 0.64	6.12 ± 0.13	742 ± 48	4388 ± 519	12924 ± 1042
Gly	0.25 ± 0.01	0.19 ± 0.01	0.75 ± 0.07	1.15 ± 0.41	4.70 ± 1.09	ND[b]
Gln	1.07 ± 0.09	0.68 ± 0.14	10.1 ± 1.5	92.1 ± 9.9	905 ± 157	1699 ± 286
His	0.21 ± 0.02	0.27 ± 0.04	0.97 ± 0.12	12.3 ± 1.3	61.4 ± 1.8	195 ± 13
Thr	0.22 ± 0.06	0.57 ± 0.04	0.78 ± 0.09	5.82 ± 0.42	17.3 ± 1.8	45.5 ± 5.7
Ala	1.25 ± 0.01	0.63 ± 0.11	5.47 ± 0.78	39.2 ± 3.2	76.1 ± 7.1	174 ± 13
Arg	4.29 ± 0.25	7.09 ± 1.11	15.2 ± 0.6	141 ± 14	755 ± 53	2326 ± 193
Pro	3.12 ± 0.08	4.77 ± 0.95	6.95 ± 1.00	13.3 ± 2.9	32.2 ± 4.0	38.7 ± 7.6
Tyr	0.47 ± 0.04	0.92 ± 0.13	1.39 ± 0.19	14.2 ± 2.1	7.68 ± 0.25	19.0 ± 7.6
Val	0.39 ± 0.01	0.95 ± 0.06	1.50 ± 0.07	39.7 ± 0.5	140 ± 2	254 ± 12
Met	0.08 ± 0.01	0.07 ± 0.01	0.25 ± 0.02	1.68 ± 0.10	2.52 ± 0.71	4.85 ± 1.28
Cys	BDL[a]	BDL	BDL	0.15 ± 0.01	14.6 ± 7.7	66.7 ± 7.4
Ile	0.16 ± 0.01	0.46 ± 0.01	0.46 ± 0.04	13.9 ± 1.1	32.4 ± 0.9	63.4 ± 3.3
Leu	0.11 ± 0.01	0.45 ± 0.07	0.57 ± 0.05	14.9 ± 2.5	26.5 ± 5.5	35.5 ± 2.3
Phe	0.21 ± 0.01	0.53 ± 0.19	0.40 ± 0.03	6.92 ± 0.17	15.4 ± 1.7	17.0 ± 1.2
Trp	0.16 ± 0.01	0.17 ± 0.02	0.19 ± 0.02	3.18 ± 0.10	17.2 ± 0.9	45.9 ± 3.5
Lys	0.10 ± 0.01	0.10 ± 0.02	0.28 ± 0.09	2.27 ± 0.25	3.19 ± 0.69	12.7 ± 3.1
Total	23.9 ± 0.3	38.5 ± 4.2	91.1 ± 2.0	1215 ± 57	6699 ± 667	18330 ± 1423
Total protein	210 ± 10	210 ± 10	225 ± 8	500 ± 16	1306 ± 65	2079 ± 68

[a] BDL: below detection limit

[b] ND: not determined

TABLE 3.4 Composition of the soluble amino acid pools in megagametophytes during post-embryonic development.

Amino acid values are expressed as nmol megagametophyte^{-1}. Total protein is expressed as µg megagametophyte^{-1}. Each value is the mean ± SE of three independent replicates.

Amino Acid	Mature	35 DAI_2	3 DAI_{30}	6 DAI_{30}	9 DAI_{30}	12 DAI_{30}
Asp	7.14 ± 0.71	8.53 ± 1.09	24.1 ± 0.9	16.7 ± 4.0	20.7 ± 4.1	17.6 ± 2.7
Glu	12.1 ± 1.4	21.7 ± 3.9	52.8 ± 1.2	73.7 ± 3.1	80.1 ± 10.7	50.4 ± 13.2
Ser	3.10 ± 0.54	1.85 ± 0.12	6.76 ± 0.46	14.7 ± 1.8	22.7 ± 1.8	49.7 ± 4.3
Asn	14.6 ± 3.5	10.3 ± 0.1	10.8 ± 1.0	27.7 ± 3.0	35.6 ± 2.6	45.3 ± 5.4
Gly	0.90 ± 0.09	0.86 ± 0.22	1.71 ± 0.21	4.69 ± 0.92	8.74 ± 0.30	22.8 ± 1.9
Gln	4.82 ± 0.61	2.87 ± 0.57	14.8 ± 4.8	34.2 ± 5.3	28.5 ± 6.9	77.2 ± 4.3
His	0.72 ± 0.18	0.91 ± 0.14	1.85 ± 0.15	5.99 ± 0.56	11.6 ± 0.9	21.3 ± 1.8
Thr	1.03 ± 0.30	1.66 ± 0.59	1.94 ± 0.13	6.83 ± 0.80	12.6 ± 0.6	30.4 ± 1.0
Ala	1.75 ± 0.57	1.60 ± 0.13	10.8 ± 4.6	14.0 ± 3.8	12.8 ± 0.4	26.7 ± 3.1
Arg	8.94 ± 1.60	10.9 ± 0.3	19.4 ± 2.3	73.0 ± 2.7	213 ± 14	241 ± 16
Pro	2.41 ± 0.76	2.55 ± 0.24	9.38 ± 0.64	26.5 ± 2.0	47.9 ± 3.6	114 ± 7
Tyr	1.76 ± 0.35	2.33 ± 0.14	5.42 ± 0.45	15.3 ± 3.4	20.1 ± 0.4	28.4 ± 2.3
Val	1.94 ± 0.26	2.64 ± 0.24	5.99 ± 0.27	15.4 ± 2.8	21.6 ± 1.1	44.7 ± 2.6
Met	0.50 ± 0.02	0.42 ± 0.03	1.37 ± 0.25	2.77 ± 0.06	3.19 ± 0.13	5.40 ± 0.41
Cys	BDL	BDL	BDL	BDL	0.73 ± 0.40	1.44 ± 0.16
Ile	1.07 ± 0.12	1.85 ± 0.18	2.72 ± 0.02	6.29 ± 0.35	11.0 ± 0.4	21.8 ± 0.7
Leu	1.09 ± 0.09	2.57 ± 0.32	3.92 ± 0.25	8.35 ± 0.43	18.4 ± 1.8	26.7 ± 1.0
Phe	0.63 ± 0.10	0.95 ± 0.07	1.37 ± 0.36	5.01 ± 0.46	12.8 ± 0.4	15.4 ± 0.8
Trp	1.02 ± 0.10	1.37 ± 0.11	1.57 ± 0.09	4.55 ± 0.67	13.5 ± 0.5	16.0 ± 3.5
Lys	0.37 ± 0.02	0.47 ± 0.06	0.53 ± 0.09	1.26 ± 0.26	2.74 ± 0.15	5.71 ± 0.87
Total	65.9 ± 6.0	76.4 ± 4.4	177 ± 10	344 ± 17	598 ± 32	862 ± 61
Total protein	1816 ± 78	1772 ± 76	1797 ± 93	1904 ± 115	1489 ± 63	831 ± 83

[a] BDL: below detectable limits

following radicle emergence. The same was also true, although to a lesser degree, for the megagametophyte. Levels of glutamate increased in the seedling through all stages examined, but represented a smaller percentage of the free amino acid pool in 9 DAI_{30} and 12 DAI_{30} seedlings than during the earlier stages of development. Levels of arginine and glutamine continued to increase in 6 DAI_{30}, 9 DAI_{30}, and 12 DAI_{30} seedlings; these amino acids were the second and third most abundant amino acids, respectively, in seedlings from these stages of development (Table 3.3). Levels of asparagine, which was not especially prevalent in the earlier stages of development, escalated following radicle emergence to make it the most abundant amino acid in 6 DAI_{30}, 9 DAI_{30}, and 12 DAI_{30} seedlings. Asparagine made up 70% of the free amino acids in the 12 DAI_{30} seedling, while arginine accounted for 13% of the free amino acid pool in 12 DAI_{30} seedlings. Glycine concentrations were not determined for 12 DAI_{30} seedlings because the very large asparagine peak did not permit satisfactory resolution of the small glycine peak.

Arginine constituted the highest percentage of the free amino acid pool in 9 DAI_{30} and 12 DAI_{30} megagametophytes (Table 3.4): arginine made up approximately 28% of the free amino acid pool in the 12 DAI_{30} megagametophyte, a stage at which storage protein breakdown is well advanced (Fig. 3.4). Glutamate was also a major amino acid in the megagametophyte at 6 DAI_{30}, 9 DAI_{30}, and 12 DAI_{30}, even though levels of this amino acid declined from 9 DAI_{30} to 12 DAI_{30}. Levels of proline and glutamine increased quite substantially from 9 DAI_{30} to 12 DAI_{30}; respectively, these amino acids represented the second and third largest component of the free amino acid pool in the 12 DAI_{30} seedlings. Asparagine did not constitute a large proportion of the amino acid pool in the megagametophyte.

Arginine represented a larger percentage of the free amino acid pools in the megagametophyte than in the seedling; however, because of the much larger pool size of the seedling compared to the megagametophyte, the seedling contained more arginine than the megagametophyte, even on a per mg dry weight basis (Fig. 3.10).

3.3.3 Seedling-Megagametophyte Interactions

While soluble amino acid levels in seedlings cultured with an intact megagametophyte increased 62-fold after 10 days in culture, levels in isolated seedlings increased by only 8-fold during the same period (Fig. 3.11 A). Like seedlings from intact seeds (Fig. 3.9 B), isolated seedlings accumulated more soluble amino acids in root poles than shoot poles (Fig. 3.11 B).

Megagametophyte halves cultured without seedlings exhibited a 28-fold increase in soluble amino acids, while amino acid pools in intact megagametophytes cultured with seedlings increased by 8-fold (Fig. 3.12 A). Soluble amino acid accumulation by isolated megagametophyte halves cultured with the cut surface proximal or distal to the agar was nearly identical (data not shown). In the absence of the seedling, isolated megagametophyte halves exported amino acids into 0.8% (w/v) agar in a pattern similar to the accumulation of amino acids by seedlings cultured with an intact megagametophyte (Fig. 3.12 B).

Megagametophytes that were gently removed from 9 to 10 DAI_2 seedlings and cultured on agar or other solid support were also capable of exporting amino acids into the vacant corrosion cavity (Fig 3.13). This exudate contained a high concentration of free amino acids (128.8 ± 8.9 nmol μL^{-1}) (Table 3.5). Glutamine was by far the most abundant amino acid, accounting for 53% of the total amino acids in the exudate. Alanine

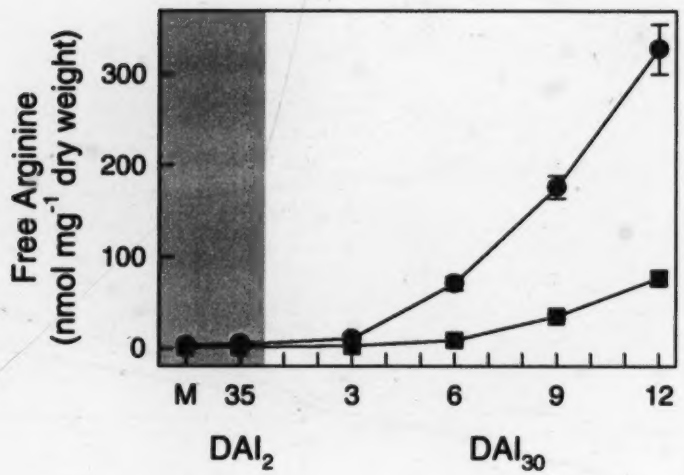


FIGURE 3.10 Free arginine content of seedlings (●) and megagametophytes (■) from intact seed during post-embryonic development, determined by HPLC. Values are the mean ± SE of three independent replicates for both arginine and dry weight determinations. M = mature desiccated seed.

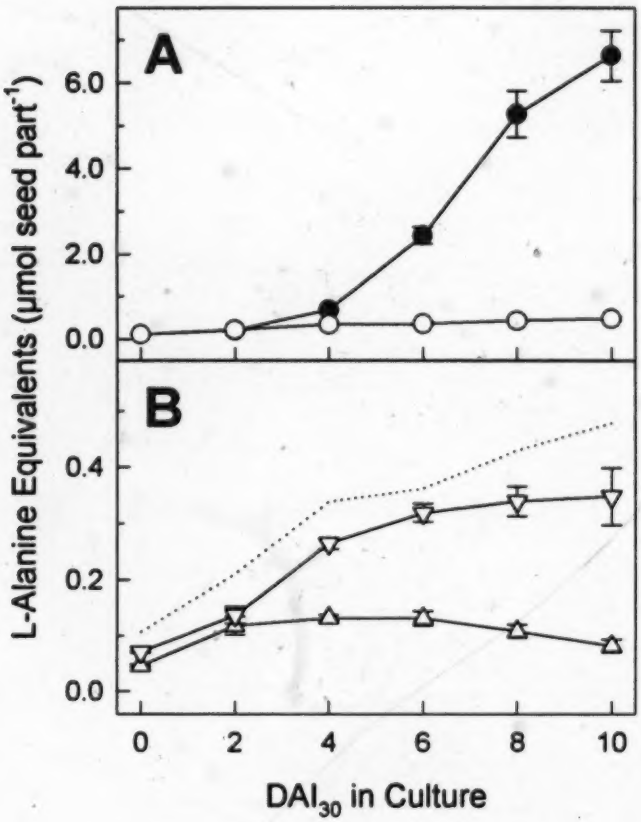


FIGURE 3.11 The effect of the megagametophyte on soluble amino acid accumulation by the seedling, determined by ninhydrin assay. Seed parts excised from 35 DAI_2 seeds were cultured on 3 % (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B. **A**, Seedlings cultured with (●) or without (○) intact megagametophytes. **B**, Seedlings cultured without megagametophytes, bisected into shoot poles (△) and root poles (▽) prior to assaying. Amino acid accumulation by isolated whole seedlings is shown for comparison (·····). Values are a mean ± SE of three independent replicates, analyzed in triplicate.

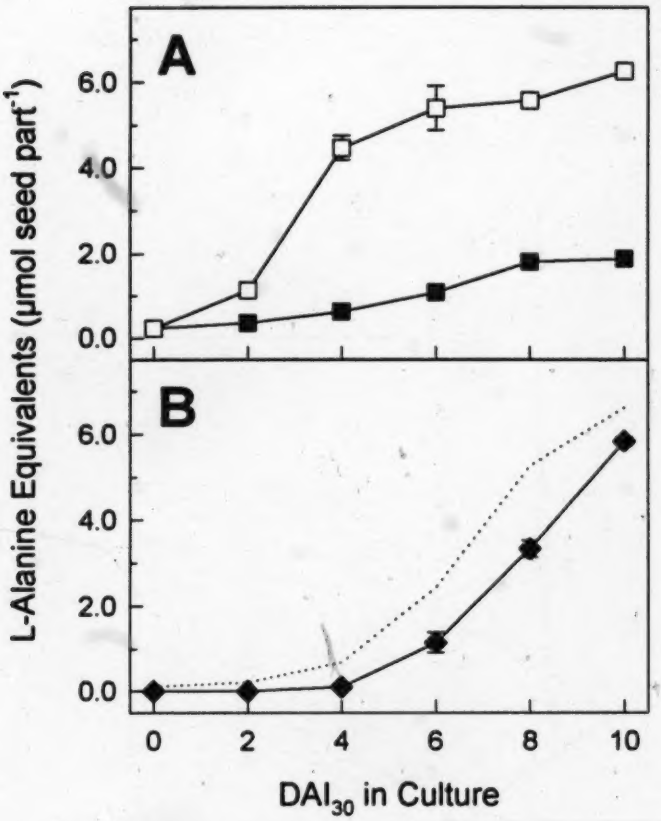


FIGURE 3.12 The effect of the seedling on soluble amino acid accumulation by the megagametophyte, determined by ninhydrin assay. Seed parts excised from 35 DAI_2 seeds were cultured on 3 % (w/v) agar (except where noted), with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B. **A**, Intact megagametophytes cultured with seedlings (■); isolated megagametophyte halves cut along a median longitudinal sagittal plane and placed with the cut surface proximal to the surface of the agar (□). **B**, Amino acid export from isolated megagametophyte halves into 0.8 % (w/v) agar (◆). Amino acid accumulation by seedlings cultured with intact megagametophytes (·····) is shown for comparison. Values are a mean ± SE of three independent replicates, analyzed in triplicate.

FIGURE 3.13 Production of exudate by intact megagametophytes removed from 9 to 10 DAI_{30} seedlings and cultured overnight on 3 % (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B. Scale: 10 mm = 700 µm.

TABLE 3.5 Amino acid composition of exudate collected from the corrosion cavity of intact, isolated 9 to 10 DAI_{30} megagametophytes.

Values are the mean ± SE of three independent replicates.

Amino Acid	nmol μL^{-1} exudate
Asp	0.289 ± 0.025
Glu	2.16 ± 0.22
Ser	5.16 ± 0.40
Asn	6.42 ± 0.43
Gly	0.680 ± 0.027
Gln	71.1 ± 4.9
His	1.52 ± 0.10
Thr	0.915 ± 0.037
Ala	8.89 ± 0.79
Arg	8.65 ± 0.47
Pro	3.12 ± 0.28
Tyr	0.600 ± 0.060
Val	5.73 ± 0.37
Met	2.16 ± 0.17
Cys	0.095 ± 0.006
Ile	2.68 ± 0.19
Leu	4.75 ± 0.35
Phe	2.36 ± 0.13
Trp	0.562 ± 0.038
Lys	0.606 ± 0.026

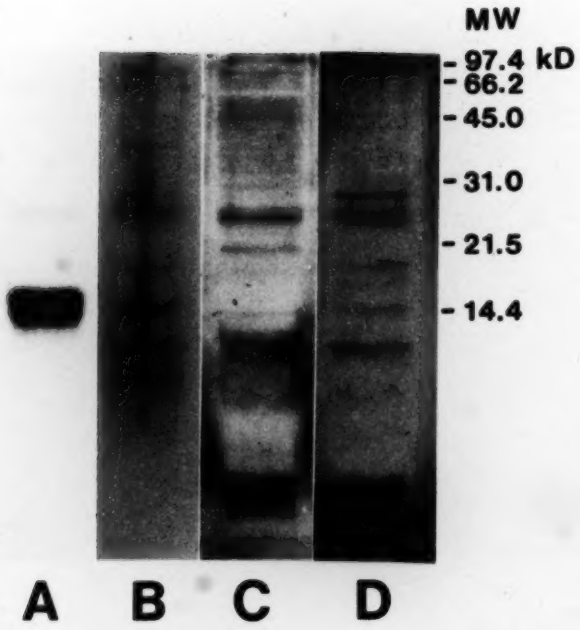


FIGURE 3.14 Exudate proteins. Exudate was collected from megagametophytes removed intact from 9 to 10 DAI_{30} seedlings, positioned on agar such that the micropylar end of the megagametophyte was distal to the agar, and cultured overnight. Coomassie blue-stained (lanes A and C) and silver-stained (lanes B and D) 18% SDS-PAGE profiles in the absence (lanes A and B) and presence (lanes C and D) of β-mercaptoethanol. Molecular weight masses are indicated to the right of the gel.

and arginine were the second and third most abundant amino acids in the exudate, representing 6.7% and 6.5%, respectively, of the amino acid content. Proteins were also present in low abundance in the 9 to 10 DAI_{30} megagametophyte exudate (Fig. 3.14).

3.4 Arginase

3.4.1 Arginase Activity During Post-Embryonic Development

Arginase activity was measured by colorimetric determination of the amount of urea produced by a Tris-soluble tissue extract in the presence of excess arginine. As a precautionary measure, a few samples were assayed in the presence of the urease inhibitor PPD to test whether urease present in the extract might break down the urea evolved by the activity of arginase, leading to an underestimate of arginase activity (Appendix 3). There was no significant difference in levels of urea determined in the presence or absence of PPD in these samples, at $\alpha = 0.05$. Since the pH optima for ureases in other species (see Torisky and Polacco, 1990) are lower than the pH optimum for loblolly pine of pH 9.7 (Appendix 3), urease activity may have been reduced to below detectable limits in the assay mixture.

When expressed as specific activity, arginase activity increased 6-fold in the seedling following imbibition, and peaked at 8 DAI_{30}. A 4-fold increase in arginase specific activity was observed in the megagametophyte following seed imbibition. Maximum specific activity of arginase occurred at 6 DAI_{30} in the megagametophyte, although activity was relatively constant in this tissue from 35 DAI_{30} onwards (Fig. 3.15 A). Peak arginase specific activity was 3.6-fold greater in the seedling than in the megagametophyte. When expressed on a per seed part basis, there was a 43-fold increase in arginase activity in the seedling from mature non-stratified seed to peak activity at

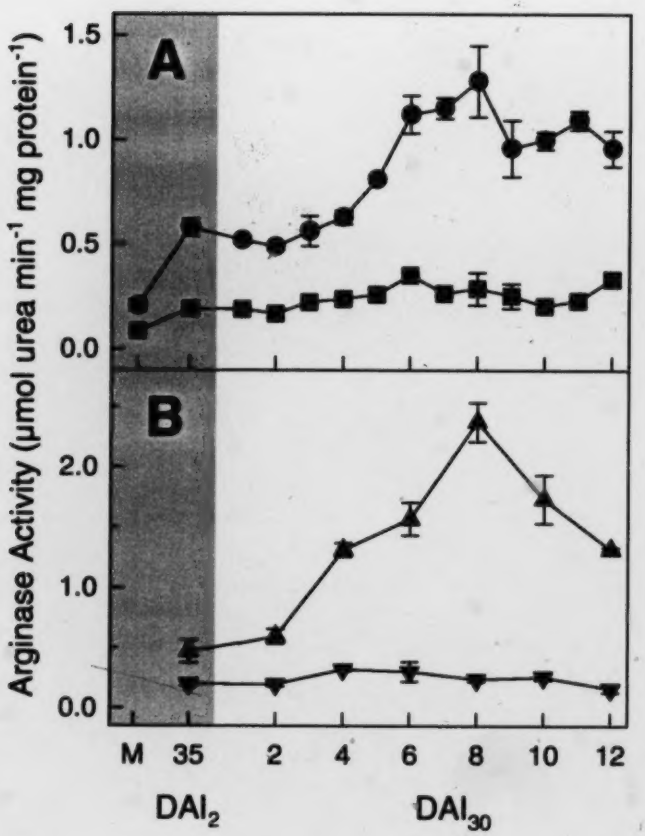


FIGURE 3.15 Arginase specific activity (µmol urea min^{-1} mg^{-1} protein) during post-embryonic development. **A**, Arginase activity in seedlings (●) and megagametophytes (■) from intact seed, and **B**, in seedlings from intact seed, bisected into root pole (▼) and shoot pole (▲) segments prior to assaying. Values are a mean ± SE of three independent replicates, analyzed in triplicate. M, mature desiccated seed.

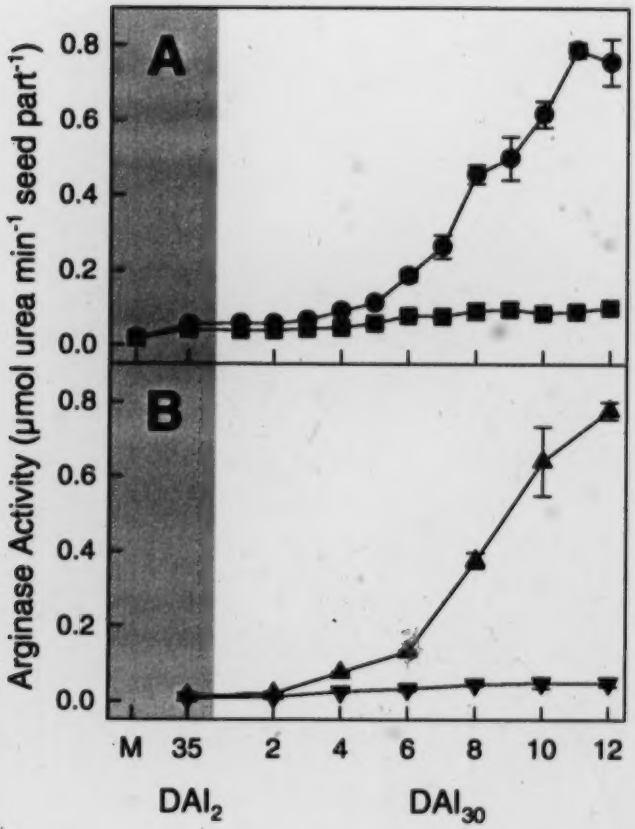


FIGURE 3.16 Arginase activity (µmol urea min^{-1} seed $part^{-1}$) during post-embryonic development. **A**, Arginase activity in seedlings (●) and megagametophytes (■) from intact seed, and **B**, in seedlings from intact seed, bisected into root pole (▼) and shoot pole (▲) segments prior to assaying. Values are a mean ± SE of three independent replicates, analyzed in triplicate. **M**, mature desiccated seed.

11 DAI_{30}; arginase activity in the megagametophyte increased only 6-fold following imbibition, reaching a peak at 12 DAI_{30} (Fig. 3.16 A). The increase in arginase activity in the seedling coincided with the accumulation of soluble amino acids in the seedling (Fig. 3.9), as well as with the accumulation of free arginine (Fig. 3.10).

In 35 DAI_2 embryos, 70% of the total specific activity of arginase was located in the shoot pole segment. At 8 DAI_{30}, 91% of the specific arginase activity of the seedling was found in the shoot pole segment (Fig. 3.15 B). On a per seed part basis, the percentage of arginase activity localized in the shoot pole segment increased from 63% in 35 DAI_2 embryos to 94% in 12 DAI_{30} seedlings (Fig. 3.16 B). On a per seed part basis, virtually all of the arginase activity in the shoot pole of 12 DAI_{30} seedlings was located in the cotyledons (Table 3.6). When expressed on a per mg protein basis, arginase activity in the cotyledons was 2.2-fold greater than arginase activity in the epicotyl.

Levels of urea, the product of arginase-mediated arginine breakdown, were greater in the seedling than in the megagametophyte at all stages of development (Fig. 3.17). Urea was quantified by colorimetric assay of heat-inactivated Tris-soluble extracts, as described in Section 2.12. The level of urea per seedling increased slightly during stratification, declined to pre-stratification levels during germination, then rose after 6 DAI_{30} (Fig. 3.17 B). Expressed on a per mg dry weight basis, the proportion of urea in seedlings increased somewhat during the course of stratification, and then fell to pre-stratification levels by 6 DAI_{30} (Fig. 3.17 A). Urea levels then rose between 6 and 8 DAI_{30}, and remained relatively constant until 12 DAI_{30}. In the megagametophyte, the level of urea per seed part remained steady through all stages examined (Fig. 3.17 B). The proportion of urea per mg dry weight in megagametophytes remained constant until the

TABLE 3.6 Localization of arginase activity within 12 DAI_{30} seedlings.

Organ	Arginase Activity	
	µmol urea min^{-1} seed $part^{-1}$	µmol urea min^{-1} mg^{-1} protein
Epicotyl[a]	0.0026 ± 0.0004	0.696 ± 0.262
Cotyledons[a]	0.7432 ± 0.0361	1.522 ± 0.060
Hypocotyl + Radicle[b]	0.0487 ± 0.0103	0.158 ± 0.030

[a] Values are the means of two independent replicates ± SE

[b] Values are the means of three independent replicates ± SE

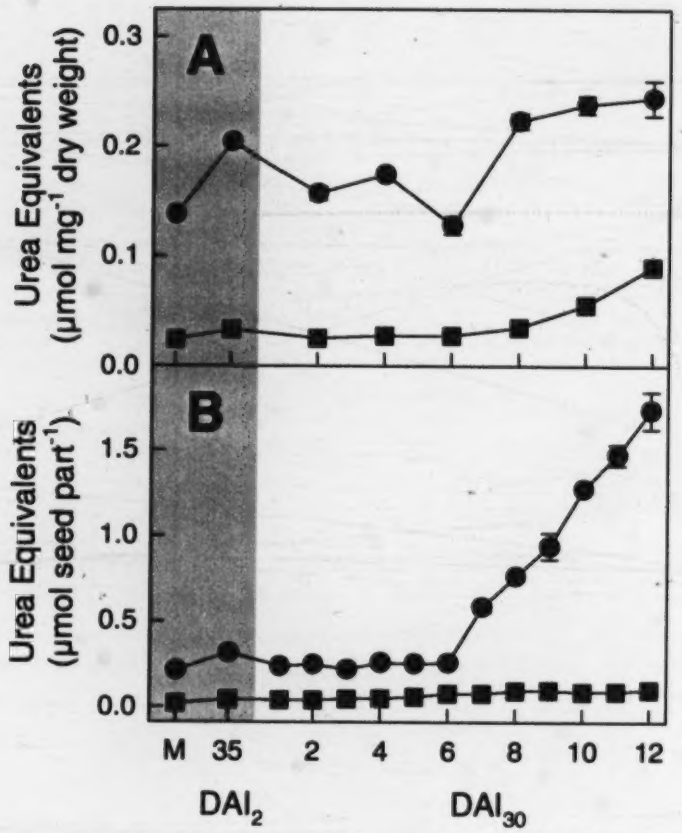


FIGURE 3.17 Quantitative changes in urea levels in seedlings (●) and megagametophytes (■) of intact seeds during post-embryonic development. **A**, Urea levels expressed as µmol urea equivalents mg^{-1} dry weight. **B**, Urea levels expressed as µmol urea equivalents seed $part^{-1}$. Values are the mean ± SE of three independent replicates. M, mature desiccated seed.

latter stages of seedling growth, then rose slightly (Fig. 3.17 A). At 12 DAI_{30}, levels of urea per mg dry weight were 2.7-fold greater in the seedling than in the megagametophyte.

3.4.2 Effect of Blocking Urease-Mediated Urea Metabolism

Levels of urea per mg dry weight were higher in seedlings with intact megagametophytes cultured on Kimpak with 1.1 mM of the urease inhibitor PPD than in seedlings cultured on Kimpak wetted with water (Fig. 3.18). The increase in urea pool size in seedlings in which urease activity was inhibited demonstrates that urease is normally active in loblolly pine during early seedling growth.

The development of the seedlings with intact megagametophytes grown in the presence of PPD was also compromised (Fig. 3.19). After 8 days in culture, the average radicle length of seedlings cultured on Kimpak saturated with 1.1 mM PPD was 13 ± 1 mm (mean ± SE, n=169), while that of seedlings cultured on water-saturated Kimpak was 60 ± 3 mm (mean ± SE, n=110) (Fig. 3.19 A). The dry weight of PPD-treated seedlings after 8 days in culture was 2.6 ± 0.2 mg per seedling (mean ± SE, n=7), whereas the dry weight of the control seedlings was 7.8 ± 0.2 mg per seedling (mean ± SE, n=7) (Fig. 3.19 B). The accumulation of Tris buffer-soluble proteins was also lower in seedlings cultured with PPD than in seedlings cultured with water (Fig. 3.19 C). PPD-treated seedlings contained 151 ± 6 µg soluble protein per seedling (mean ± SE, n=6) after 8 days in culture, while control seedlings contained 632 ± 52 µg soluble protein per seedling (mean ± SE, n=6). On a per mg dry weight basis, PPD-treated seedlings and control seedlings contained 72.6 ± 9.2 and 97.7 ± 8.8 µg soluble protein per mg dry weight, respectively, after 8 days in culture.

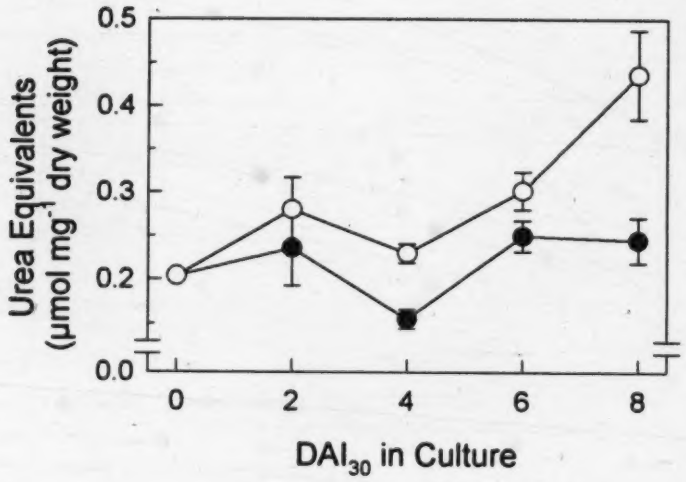


FIGURE 3.18 Effect of the urease inhibitor PPD on urea accumulation by seedlings. Seedlings with intact megagametophytes from 35 DAI_2 seeds were cultured on Kimpak moistened with water (●) or 1.1 mM PPD (○); 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B were also included. Values are the mean ± SE of three independent replicates for both urea and dry weight determinations.

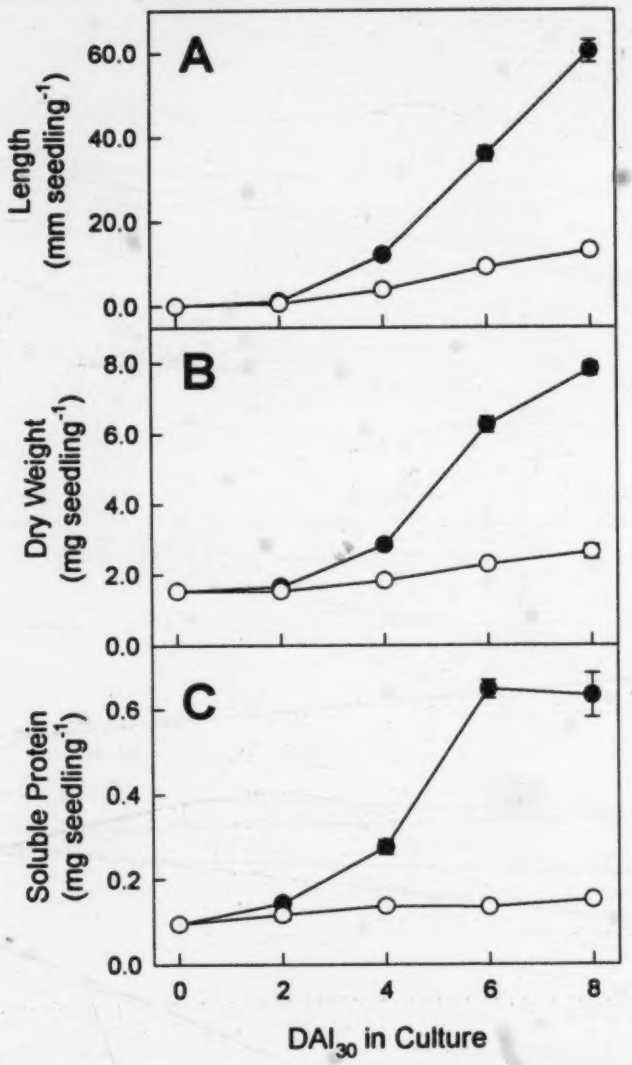


FIGURE 3.19 Effect of the urease inhibitor PPD on seedling growth. Seedlings with intact megagametophytes from 35 DAI_2 seeds were cultured on Kimpak moistened with water (●) or 1.1 mM PPD (○); 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B were also included. **A**, Total seedling length (n=110 to 266). **B**, Seedling dry weight (n=7). **C**, Seedling Tris buffer-soluble protein (n=6). Values represent the means ± SE.

Arginase activity was markedly reduced in seedlings with intact megagametophytes that were cultured with 1.1 mM PPD (Fig. 3.20). Storage protein breakdown in the megagametophyte, estimated by quantifying buffer-insoluble protein, was also affected by PPD. Levels of buffer-insoluble proteins in PPD-treated megagametophytes with seedlings remained high, while buffer-insoluble proteins in the control megagametophytes with seedlings decreased in the characteristic pattern associated with storage protein breakdown (Fig. 3.21).

3.4.3 Seedling-Megagametophyte Interactions

Arginase activity was substantially lower in isolated seedlings cultured from 35 DAI_2 seeds than in seedlings cultured with intact megagametophytes, but with all other seed tissues removed (Fig. 3.22). Removal of the megagametophyte from 4 DAI_{30} or 9 DAI_{30} seedlings grown from intact seeds also resulted in decreased arginase activity compared to seedlings cultured with intact megagametophytes (Figs. 3.23 and 3.24). The levels of arginase activity in seedlings excised from intact 4 DAI_{30} seedlings and cultured without megagametophytes was reduced after four hours of culture compared to that of seedlings excised from intact 4 DAI_{30} seedlings and cultured with intact megagametophytes, whether the data were expressed as per seed part or per mg protein (Fig. 3.23). Arginase activity was very similar in isolated seedlings removed from 4 DAI_{30} seeds and cultured on Kimpak with or without 100 mM sucrose. In contrast to isolated 4 DAI_{30} seedlings, culturing isolated 9 DAI_{30} seedlings on Kimpak with 100 mM sucrose resulted in lower levels of arginase activity than if isolated 9 DAI_{30} seedlings were cultured without sucrose (Fig. 3.24). After ten hours in culture, arginase activity in isolated 9 DAI_{30} seedlings cultured with or without sucrose was lower than that in 9 DAI_{30}

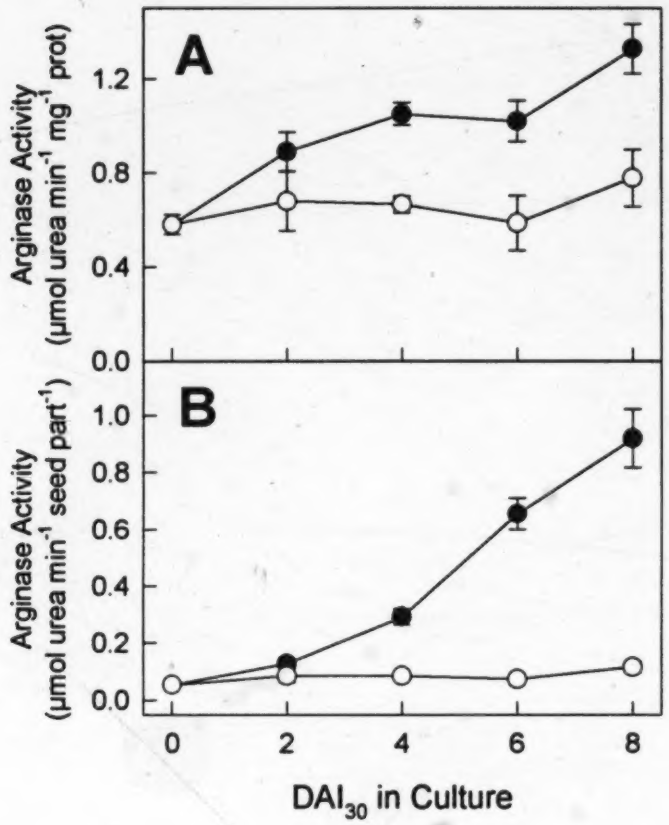


FIGURE 3.20 Effect of the urease inhibitor PPD on arginase activity in seedlings. Seedlings from 35 DAI_2 seeds were cultured with intact megagametophytes on Kimpak dampened either with water (●) or with 1.1 mM PPD (○); 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B were also included. **A**, Arginase activity expressed as µmol urea min^{-1} mg^{-1} protein. **B**, Arginase activity expressed as µmol urea min^{-1} seed $part^{-1}$. Values are the mean ± SE of three independent replicates, analyzed in triplicate.

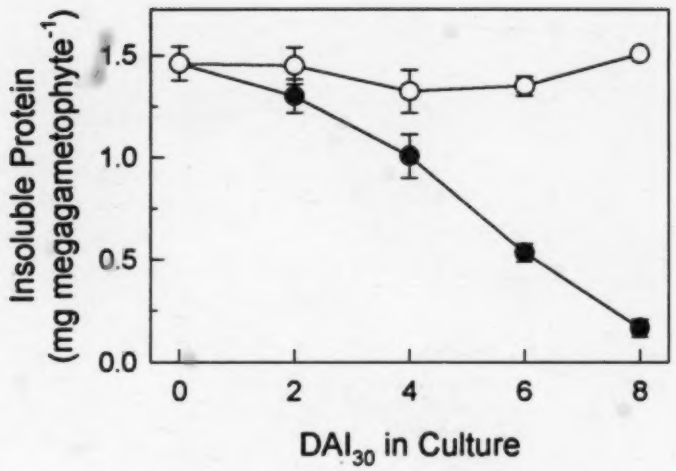


FIGURE 3.21 Effect of the urease inhibitor PPD on buffer-insoluble protein levels in the megagametophyte. Seedlings from 35 DAI_2 seeds were cultured with intact megagametophytes on Kimpak dampened either with water (●) or with 1.1 mM PPD (○); 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B were also included. Values are the mean ± SE of three independent determinations, analyzed in duplicate.

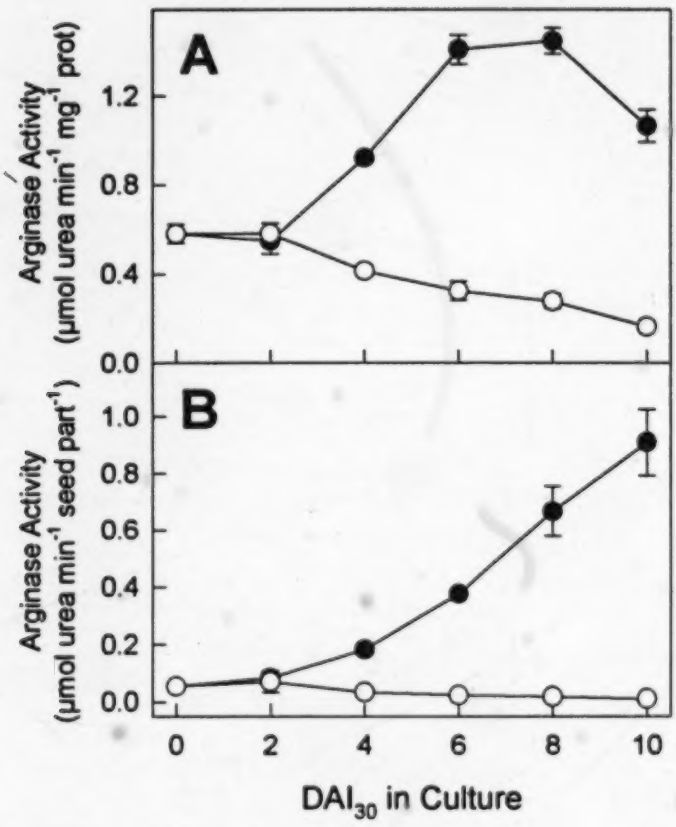


FIGURE 3.22 Effect of the megagametophyte on arginase activity in seedlings. Seedlings excised from 35 DAI_2 seeds were cultured with (●) or without (○) intact megagametophytes for up to 10 days on 3 % (w/v) agar with 60 mM sucrose, 15 µg/mL rifampicin and 2.5 µg/mL amphotericin B. **A**, Arginase activity expressed as µmol urea min^{-1} mg^{-1} protein. **B**, Arginase activity expressed as µmol urea min^{-1} seed $part^{-1}$. Values are a mean ± SE of three independent replicates, analyzed in triplicate.

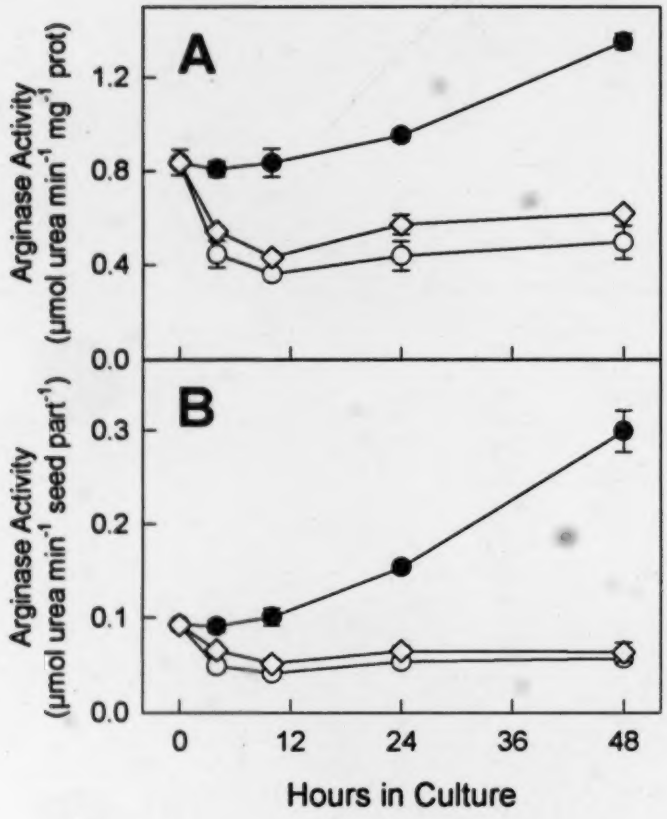


FIGURE 3.23 Effect of the megagametophyte on arginase activity in 4 DAI_{30} seedlings. Seedlings were grown as intact seeds until radicle emergence, then 4 DAI_{30} seedlings were cultured on Kimpak for up to 48 hours as seedlings with intact megagametophytes (●), or as isolated seedlings cultured with (◇) or without (○) 100 mM sucrose. **A**, Arginase activity expressed as µmol urea min^{-1} mg^{-1} protein. **B**, Arginase activity expressed as µmol urea min^{-1} seed $part^{-1}$. Values are a mean ± SE of two to five independent replicates, analyzed in triplicate.

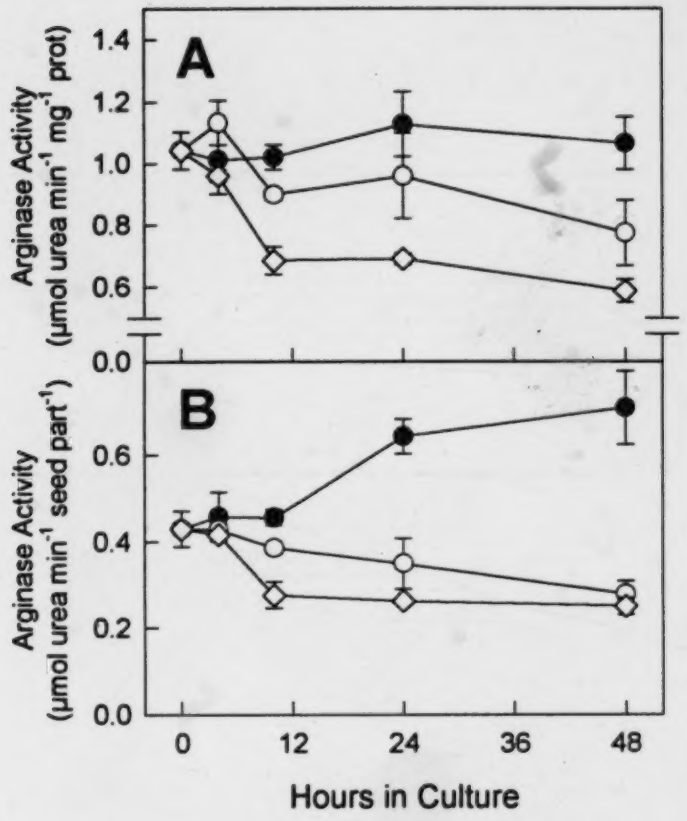


FIGURE 3.24 Effect of the megagametophyte on arginase activity in 9 DAI_{30} seedlings. Seedlings were grown as intact seeds until 9 DAI_{30}; seedlings were then excised and cultured for up to 48 hours on Kimpak with intact megagametophytes (●), or as isolated seedlings cultured with (◇) or without (○) 100 mM sucrose. **A**, Arginase activity expressed as µmol urea min^{-1} mg^{-1} protein. **B**, Arginase activity expressed as µmol urea min^{-1} seed $part^{-1}$. Values are a mean ± SE of two to three independent replicates, analyzed in triplicate.

seedlings cultured with intact megagametophytes, whether the data was expressed on a per seed part basis or a per mg protein basis.

During the first few hours of culture, levels of soluble protein in isolated seedlings excised from 4 DAI_{30} material were similar to seedlings cultured with intact megagametophytes (Fig. 3.25). However, while soluble protein levels remained relatively constant in isolated 4 DAI_{30} seedlings through 48 hours, levels increased in 4 DAI_{30} seedlings cultured with intact megagametophytes. Levels of soluble protein in isolated 4 DAI_{30} seedlings were similar regardless of treatment. Like 4 DAI_{30} seedlings, isolated seedlings excised from 9 DAI_{30} material demonstrated levels of soluble protein comparable to 9 DAI_{30} seedlings with intact megagametophytes at 4 and 10 hours of culture, but while 9 DAI_{30} seedlings with intact megagametophytes accumulated soluble protein at 24 and 48 hours of culture, soluble protein levels in isolated 9 DAI_{30} seedlings remained relatively unchanged (Fig. 3.26). Sucrose had little effect on soluble protein accumulation by isolated 9 DAI_{30} seedlings.

Arginase activity was greater in isolated 4 DAI_{30} seedlings that were cultured with 100 mM arginine than in isolated 4 DAI_{30} seedlings cultured without arginine, when the data were expressed both on a per seed part basis and a per mg protein basis (Fig. 3.27). Arginase activity in isolated 4 DAI_{30} seedlings that were cultured with 100 mM arginine and 100 mM sucrose was nearly the same as arginase activity in isolated 4 DAI_{30} seedlings cultured with 100 mM arginine and no sucrose after 4 hours (Fig. 3.28) or 10 hours (data not shown) in culture. Treatment of isolated 4 DAI_{30} seedlings with 100 mM arginine increased arginase activity to levels comparable to those of 4 DAI_{30} seedlings cultured with megagametophytes for at least the first 10 hours in culture (Fig. 3.27). However, by

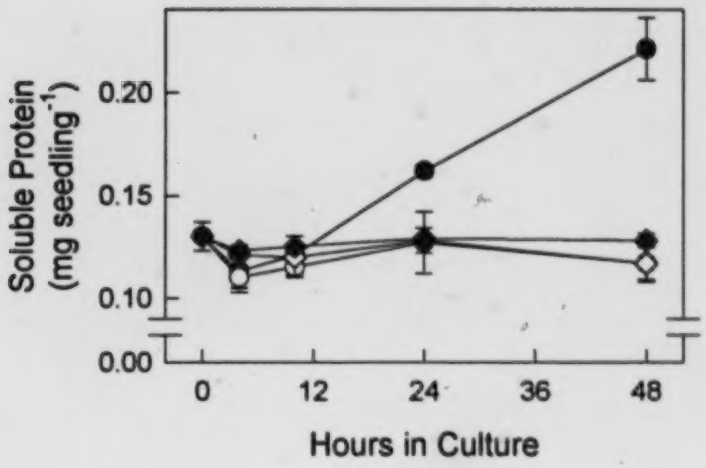


FIGURE 3.25 Soluble protein levels in 4 DAI_{30} seedlings cultured with or without megagametophytes. Seedlings were grown as intact seeds until radicle emergence, then 4 DAI_{30} seedlings were cultured for up to 48 hours on Kimpak as seedlings with intact megagametophytes (●), or as isolated seedlings cultured with 100 mM sucrose (◇), without (○) 100 mM sucrose, or with 100 mM sucrose and 100 mM arginine (◆). Values are a mean ± SE of two to five independent replicates, analyzed in duplicate.

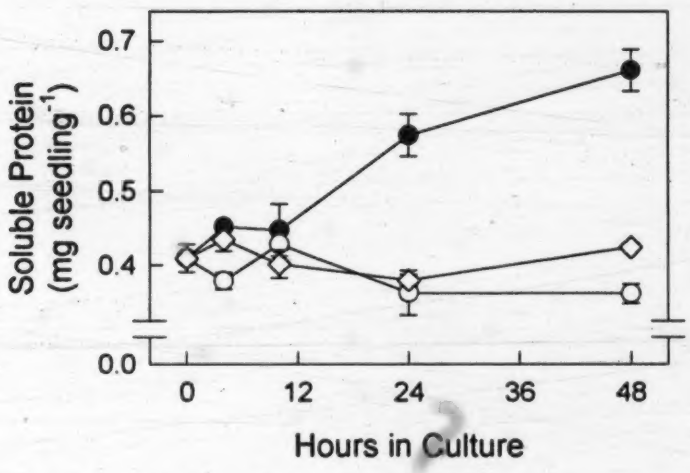


FIGURE 3.26 Soluble protein levels in 9 DAI_{30} seedlings cultured with or without megagametophytes. Seedlings were grown as intact seeds until 9 DAI_{30}; seedlings were then excised and cultured for up to 48 hours on Kimpak as seedlings with intact megagametophytes (●), or as isolated seedlings cultured with (◇) or without (○) 100 mM sucrose. Values are a mean ± SE of two to five independent replicates, analyzed in duplicate.

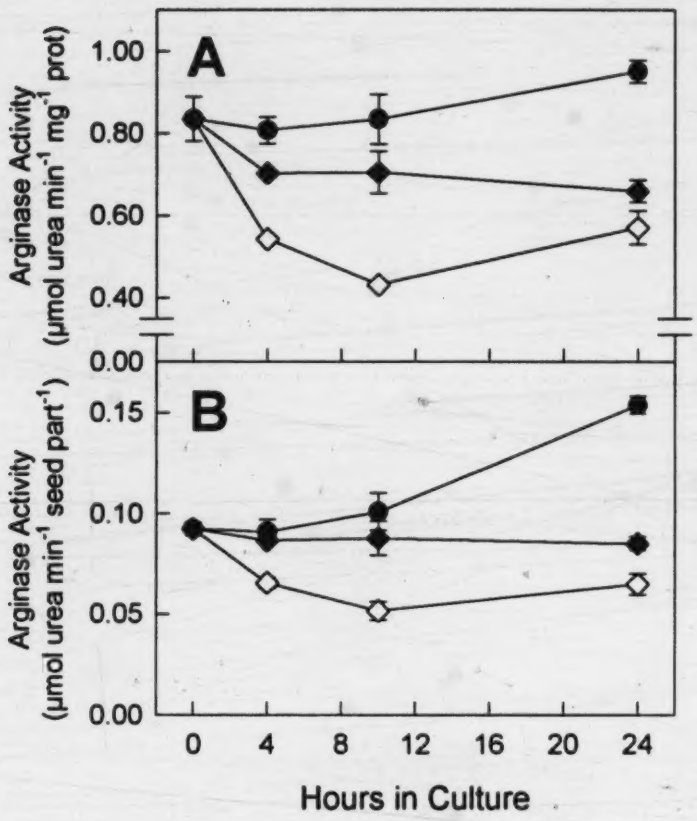


FIGURE 3.27 Effect of arginine on arginase activity in 4 DAI_{30} seedlings. Seedlings were grown as intact seeds until radicle emergence, then 4 DAI_{30} seedlings were cultured as seedlings with intact megagametophytes (●), or as isolated seedlings cultured with 100 mM sucrose and either 0 mM (◇) or 100 mM (◆) arginine. **A**, Arginase activity expressed as µmol urea min^{-1} mg^{-1} protein. **B**, Arginase activity expressed as µmol urea min^{-1} seed $part^{-1}$. Values are a mean ± SE of two to five independent replicates, analyzed in triplicate.

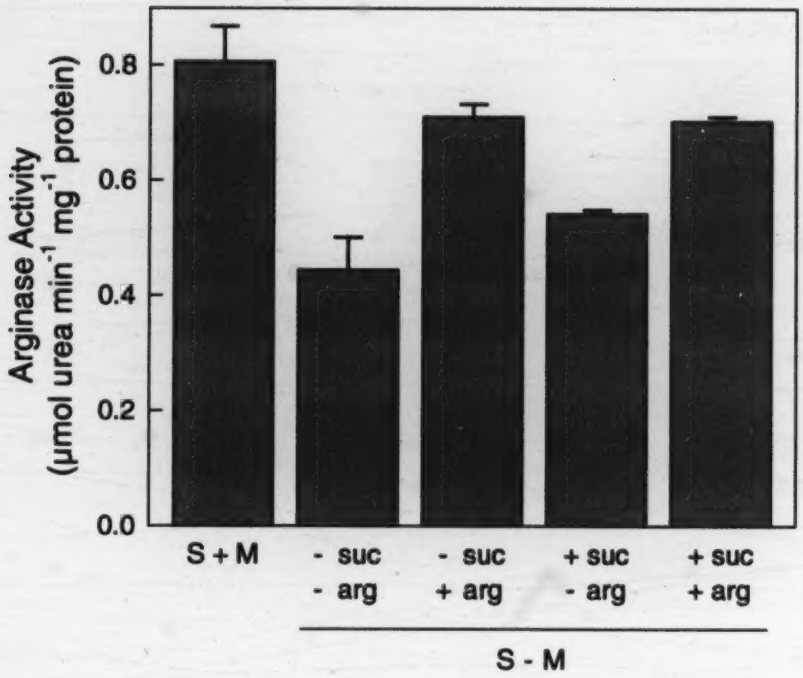


FIGURE 3.28 Effect of sucrose and arginine on arginase activity in 4 DAI_{30} seedlings. Seedlings were grown as intact seeds until radicle emergence, then 4 DAI_{30} seedlings were cultured for 4 hours as seedlings with intact megagametophytes, or as isolated seedlings cultured with 0 or 100 mM sucrose and with 0 or 100 mM arginine. Arginase activity is expressed as µmol urea min^{-1} mg^{-1} protein. Values are a mean ± SE of two independent replicates, analyzed in triplicate.

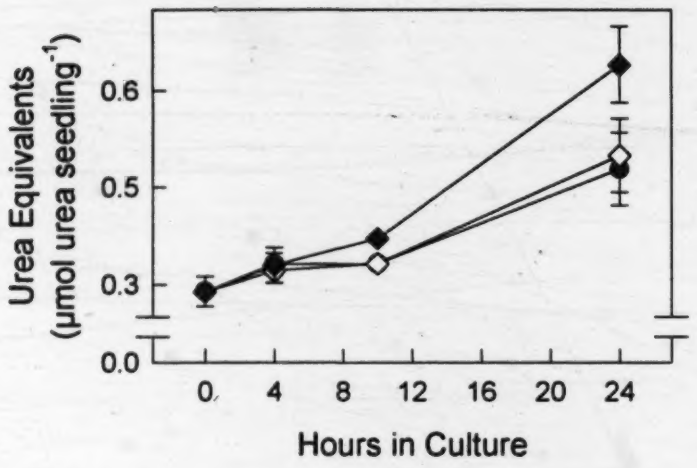


FIGURE 3.29 Effect of arginine on urea accumulation in 4 DAI_{30} seedlings. Seedlings were grown as intact seeds until radicle emergence, then 4 DAI_{30} seedlings were cultured as seedlings with intact megagametophytes (●), or as isolated seedlings cultured with 100 mM sucrose and either 0 mM (◇) or 100 mM (◆) arginine. Values are a mean ± SE of two to five independent replicates, analyzed in triplicate.

24 hours in culture, arginase activity in 4 DAI_{30} seedlings cultured with megagametophytes was approximately 40% greater than that of arginine-treated isolated 4 DAI_{30} seedlings. Levels of urea in 4 DAI_{30} seedlings cultured with 100 mM arginine were approximately 34% greater than the urea levels in 4 DAI_{30} seedlings cultured with intact megagametophytes at 24 hours of culture (Fig. 3.29).

3.5 Arginase Purification and Characterization

3.5.1 Purification of Arginase

Arginase was purified approximately 148-fold by the purification procedure summarized in Table 3.7, Figure 3.30 and Figure 3.31. Details of the protocol are provided in Section 2.13. A band migrating to 43 kD was identified as arginase, since it was the only protein detectable with silver stain in all fractions containing arginase activity. However, one contaminating band, migrating to 44 kD, had a very similar, if not identical, elution profile to the band designated as arginase, although it was always present in much lower quantities. The two bands could only be resolved using a 10% separating gel. The 43 and 44 kD proteins had the same elution profile on an FPLC Superose 8 gel filtration column (data not shown), suggesting that the holoproteins that they were derived from have a similar molecular mass. Additionally, a number of other chromatographic techniques failed to separate these bands. Although the lower band of this doublet was more likely to be arginase because it was detectable in all fractions containing arginase activity, there is not conclusive evidence to categorically state that the second band was not arginase. It is possible that both the 43 kD protein and the 44 kD protein represent arginase subunits.

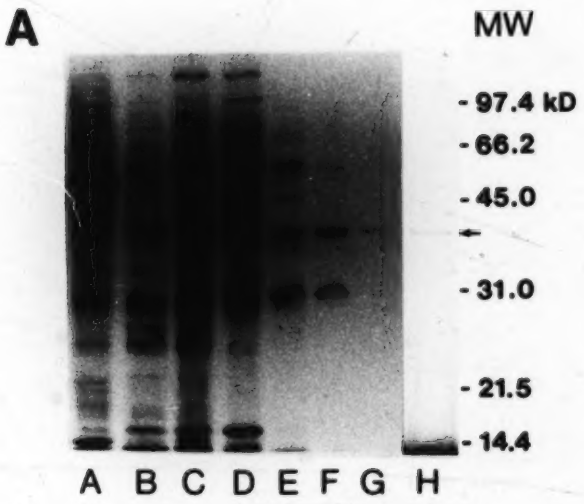
TABLE 3.7 Purification of arginase from 9 to 12 DAI_{30} loblolly pine seedling shoot poles.

Purification Step	Total Arginase Activity µmol urea min^{-1}	Protein mg	Specific Activity µmol urea min^{-1} mg^{-1} prot	Yield %	Purification -fold
Crude homogenate	3867	2062	1.88	100	-
60°C heat	3745	969	3.87	96.8	2.1
30-60% saturation ammonium sulfate[a]	1385	81.0	17.1	35.8	9.1
DE-52 anion exchange[b]	646	23.0	28.1	16.7	15.0
Matrex Green	430	4.0	107	11.1	57.3
Arginine-linked Sepharose 4B	346	2.7	130	8.9	69.1
DE-52 anion exchange	6.9	0.025	278	0.2	148

[a] Arginase activity assayed after desalting with Bio-Gel P-6DG.

[b] Arginase activity assayed after dialysis and subsequent concentration with a stirred cell concentrator.

FIGURE 3.30 Arginase purification and antibody production. **A**, SDS-PAGE profiles of pooled fractions from each stage of the purification process summarized in Table 3.7. Samples were electrophoresed on 12% separating gels under reducing conditions. Lanes A to G were stained with Coomassie blue, while Lane H was silver-stained. Lane A, crude homogenate (10 μg); Lane B, supernatant from heat-treated homogenate (10 μg); Lane C, 30-60% ammonium sulfate fraction (following desalting) (10 μg); Lane D, pooled active fractions from first DE-52 anion exchange column (8 μg); Lane E, pooled active fractions from Matrex Green affinity column (1 μg); Lane F, pooled peak activity fractions from arginine-linked Sepharose-4B affinity column (0.5 μg); Lane G, purified arginase fractions from second DE-52 anion-exchange column (0.1 μg); Lane H, silver-stained purified arginase fractions from second DE-52 anion-exchange column (0.1 μg). The arrow to the right of the gel designates arginase. Molecular mass markers are also indicated to the right of the gel. **B**, Further purification of arginase by electroelution, and immunoblot analysis using the antibodies raised to the electroeluted protein. Pooled fractions from the second DE-52 anion exchange column containing peak arginase activity were electroeluted by SDS-PAGE, using a 9.2 cm, 10.7% separating gel. Lane A, Silver-stained 12% separating gel SDS-PAGE profile of electroeluted fraction containing arginase, as well as minor amounts of a slightly higher molecular weight protein (see text for details). Lane B, Immunoblot of phosphate buffer-soluble protein from 10 DAI_{30} seedlings (25 μg). Lane C, Immunoblot of protein preparations containing approximately equal amounts of the two proteins (approximately 0.5 μg total protein). Proteins were separated on 12% separating gel SDS-PAGE, transferred to nitrocellulose, and probed with a 1:250 dilution of the antiserum raised to the electroeluted protein. Molecular mass markers are indicated to the right of Lanes A and C.


A
MW
- 97.4 kD
- 66.2
- 45.0
- 31.0
- 21.5
- 14.4
A B C D E F G H

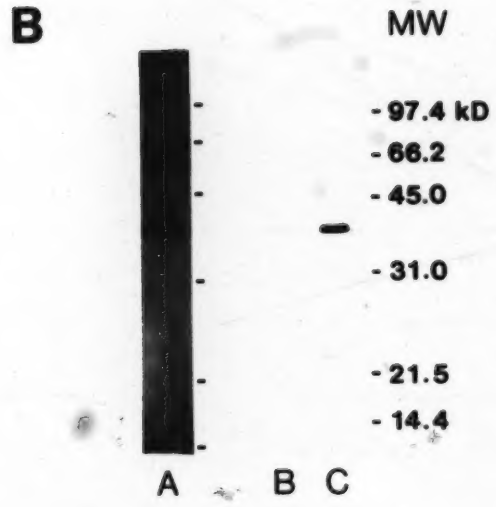
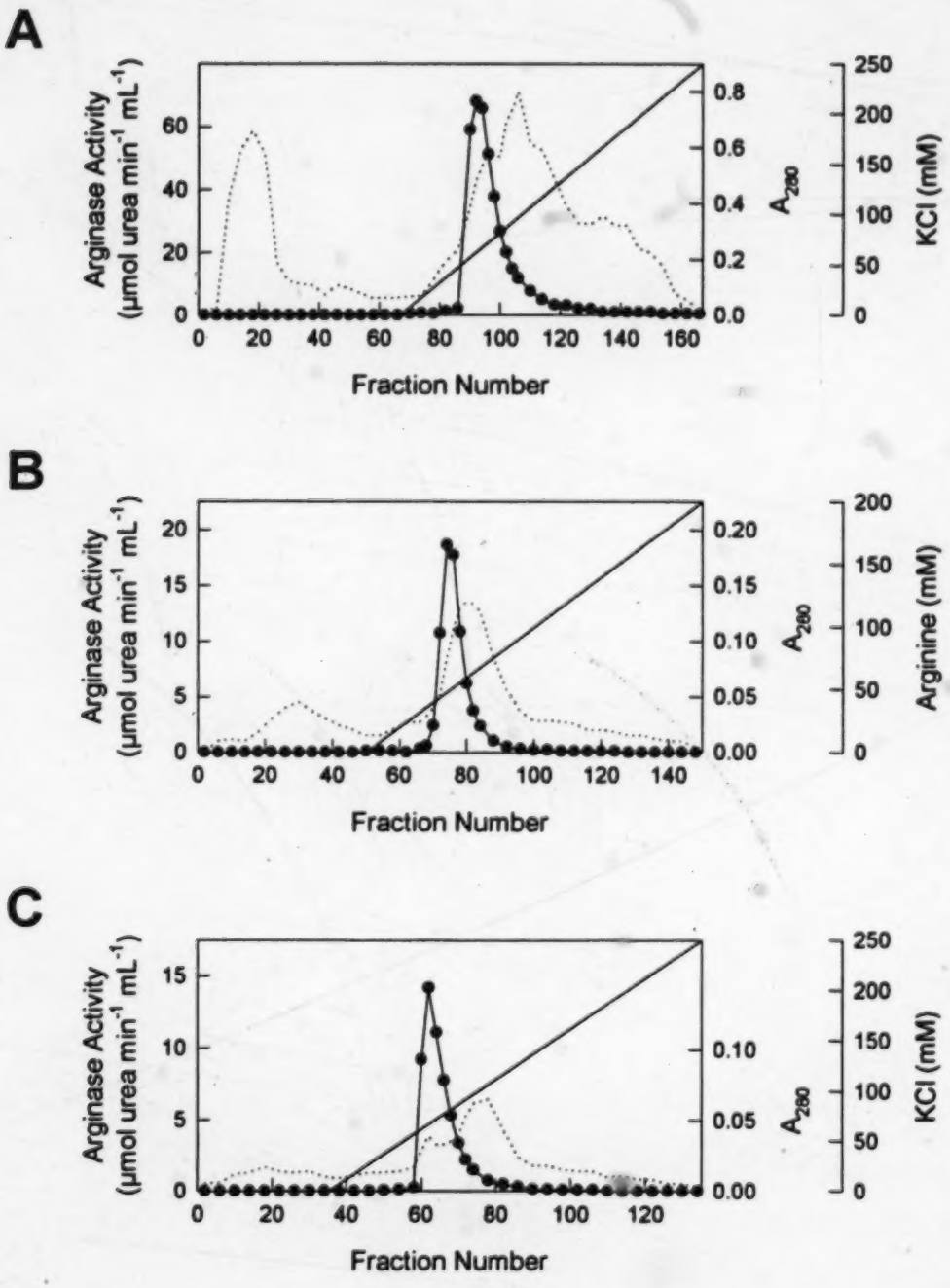

B
MW
- 97.4 kD
- 66.2
- 45.0
- 31.0
- 21.5
- 14.4
A B C

FIGURE 3.31 Purification of arginase. Elution profiles of arginase activity (●) are shown superimposed upon total protein profiles (·····). The latter were estimated by measuring absorbance values at 280 nm. **A**, The first DE-52 anion exchange column. **B**, Arginine-linked Sepharose 4B affinity chromatography. **C**, The second DE-52 anion exchange column. Solid lines indicate the gradient used to elute the proteins. The elution profile for Matrex Green is not shown, since arginase eluted in the flow-through fraction.


A
Arginase Activity (µmol urea min-1 mL-1)
A280
KCl (mM)
Fraction Number
B
Arginase Activity (µmol urea min-1 mL-1)
A280
Arginine (mM)
Fraction Number
C
Arginase Activity (µmol urea min-1 mL-1)
A280
KCl (mM)
Fraction Number

There were about eight additional minor contaminants whose elution profiles overlapped that of the protein identified as arginase. Since their elution pattern did not correspond with the arginase activity profile, these proteins were not candidates for arginase. However, their presence resulted in a consistently low yield of apparently pure arginase. The properties of arginase restricted the choice of procedures that could be used to optimize the purification protocol. For example, chromatographic procedures that resulted in the loss of soluble manganese - i.e. by precipitation, chelation, or adsorption - had to be avoided due to the concomitant loss of arginase activity (see Section 3.5.2). Enzyme activity was also adversely affected by dialysis or concentration via membrane filtration techniques.

Due to the difficulty encountered in purifying adequate quantities of the arginase holoprotein to raise anti-arginase antibodies in rabbits, electroelution was employed to generate sufficient antigen for antibody production. Fractions from the second DE-52 column that contained high arginase activity but were contaminated with other proteins were pooled and concentrated by acetone precipitation, then subjected to electroelution via SDS-PAGE using a 9.2 cm, 10.7% separating gel as described in Section 2.14. Arginase subunits were identified by comparison of molecular masses of the electroeluted proteins with that of the protein identified as arginase. The electroelution procedure yielded ca. 150 µg of purified 43 kD arginase subunits, but also contained some of the comigrating 44 kD protein (Fig. 3.30 B, Lane A). Antibodies raised against the electroeluted arginase subunits recognized both the 43 kD and 44 kD protein (Fig. 3.30 B, Lane C), but appeared to have a much higher affinity for the 43 kD protein than the 44 kD

protein when used to probe phosphate buffer-soluble extracts of 10 DAI_{30} seedlings (Fig. 3.30 B, Lane B).

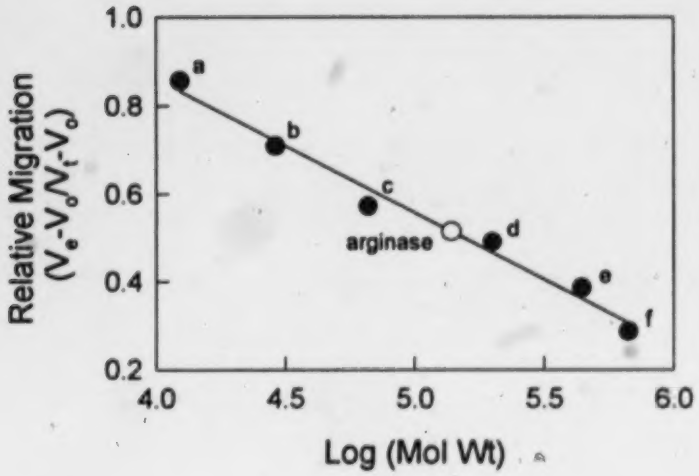
3.5.2 Characterization of Arginase

The native molecular mass of arginase was estimated to be 140 kD by gel filtration on a Superose 6 FPLC column calibrated with thyroglobulin, apoferritin, β-amylase, BSA, carbonic anhydrase, and cytochrome c (Fig.3.32 A). Based on a subunit molecular mass of 43 to 44 kD calculated from the relative migration of arginase on 10% separating gel SDS-PAGE (Fig. 3.32 B), loblolly pine arginase is predicted to be a trimeric protein.

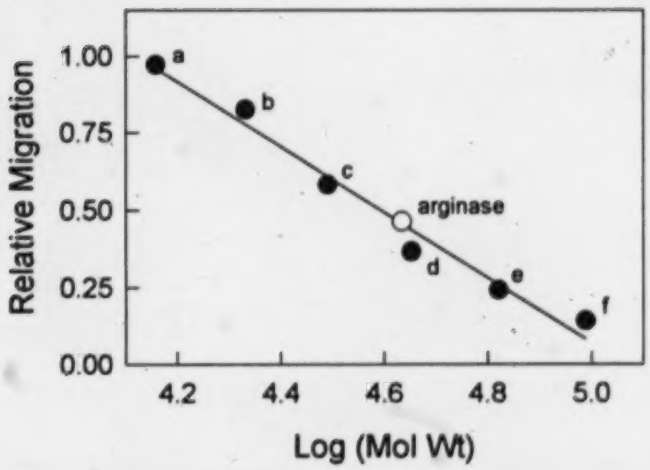
The hypothesis that loblolly pine arginase is also manganese-dependent was tested, since arginase has been found to require a manganese cofactor for activity in other bacterial, plant and animal species (Nakamura *et al.*, 1973; O'Malley and Terwilliger, 1974; Wright *et al.*, 1981; Boutin, 1982; Cavalli *et al.*, 1994; Carvajal *et al.*, 1996). Removal of Mn^{2+} from partially-purified arginase preparations by EDTA chelation, followed by exhaustive dialysis against Mn^{2+}-free buffer (50 mM Tris·HCl [pH 7.5], 10% [v/v] glycerol), reduced arginase activity to approximately 5.6 ± 0.9% of the activity in the control treatment, which was not treated with EDTA, but was dialyzed against Mn^{2+}-containing buffer (50 mM Tris·HCl [pH 7.5], 1 mM $MnCl_2$, 10% [v/v] glycerol) (Fig. 3.33). Adding $MnCl_2$ to Mn^{2+}-depleted arginase to a final concentration of 2 mM during the enzyme activation and assay steps of the activity determination protocol restored arginase activity to about 106 ± 5% of that in the control treatment. Preliminary experiments indicate that other divalent cations, such as Zn^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+}, were not able to restore the activity of inactivated arginase (data not shown).

FIGURE 3.32 Subunit molecular mass and native molecular weight of loblolly pine arginase from 9 to 12 DAI_{30} seedling shoot poles. **A**, Native molecular weight determination by FPLC on a Superose 6 gel filtration column. Molecular weight markers (●) used to calibrate the column were: a, thyroglobulin (669 kD); b, apoferritin (443 kD); c, β-amylase (200 kD); d, BSA (66 kD); e, carbonic anhydrase (29 kD); f, cytochrome c (12.4 kD). **B**, Subunit molecular mass determination by 10% SDS-PAGE. Molecular weight markers (●) used for calibration were: a, phosphorylase b (97.4 kD); b, BSA (66.2 kD); c, ovalbumin (45 kD); d, carbonic anhydrase (31 kD); e, soybean trypsin inhibitor (21.5 kD); f, lysozyme (14.4 kD).

A


1.0
0.8
0.6
0.4
0.2
Relative Migration
(V_e-V_o/V_t-V_o)
a
b
c
arginase
d
e
f
4.0
4.5
5.0
5.5
6.0
Log (Mol Wt)

B


1.00
0.75
0.50
0.25
0.00
Relative Migration
a
b
c
arginase
d
e
f
4.2
4.4
4.6
4.8
5.0
Log (Mol Wt)

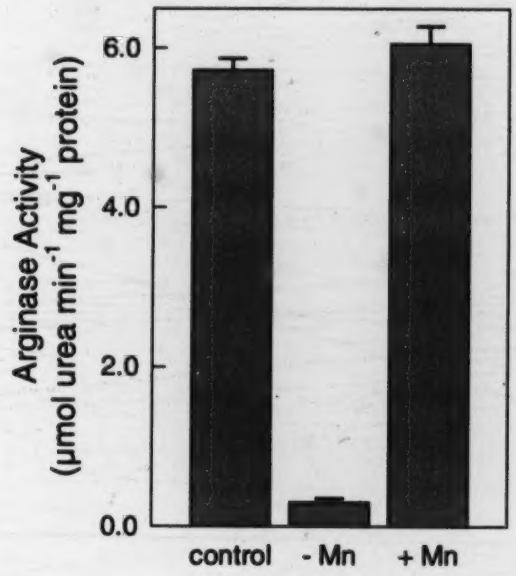


FIGURE 3.33 Effect of manganese removal and subsequent addition on arginase activity *in vitro*, expressed as µmol urea min^{-1} mg^{-1} protein. Partially purified arginase from 9-12 DAI_{30} seedling shoot poles was treated with 50 mM EDTA and dialyzed exhaustively against Mn^{2+}-free buffer to remove Mn^{2+}. Mn^{2+} was then added to one aliquot of the Mn^{2+}-free preparation to a final concentration of 2 mM for both the enzyme activation and enzyme assay portions of the procedure, while the other aliquot was activated and assayed in the absence of Mn^{2+}. (See Materials and Methods for details.) The control was not treated with EDTA, and dialyzed against Mn^{2+}- containing buffer. A total of four to five triplicate determinations of enzyme activity were made for each treatment in two separate experiments (n = 2), using the same original arginase preparation. Error bars indicate ± one SE.

4. DISCUSSION

4.1 Constraints to Seed Germination in Loblolly Pine

Intact, mature non-stratified seeds of loblolly pine exhibit slow, sporadic germination (Barnett, 1970, 1976, 1996). However, embryos excised from mature non-stratified seeds germinate readily - that is, they elongate and become green - indicating that seed dormancy is not inherent in the embryo itself. The sequential tissue removal experiments summarized in Figure 3.1 indicate that seed dormancy in loblolly pine is imposed by tissues external to the embryo. These findings are in agreement with previous studies on seed dormancy in loblolly pine (Barnett, 1970, 1976, 1996; Carpita *et al.*, 1983; Schneider and Gifford, 1994). Dormancy imposed by tissues external to the embryo is also found in several other conifer species, including slash pine (*Pinus elliottii* Engelm.) (Barnett, 1976), shortleaf pine (*Pinus echinata* Mill.) (Barnett, 1976), sugar pine (Baron, 1978), western white pine (*Pinus monticola* Dougl.) (Hoff, 1987), Norway spruce (Tillman-Sutela and Kauppi, 1995a), Scots pine (Tillman-Sutela and Kauppi, 1995b), and white spruce (Downie and Bewley, 1996).

The data in Figure 3.1 indicate that the seed coat (testa) presents the major constraint to germination of loblolly pine seeds. The seed coat of loblolly pine acts to restrict water uptake by the seed (Barnett, 1976), perhaps limiting imbibition by mechanically confining the swelling of the megagametophyte and embryo (Barnett, 1996). The seed coat has also been shown to restrict oxygen uptake by the seed (Barnett, 1976).

Chemical inhibitors of germination are not considered to be a significant factor in the seed coat-imposed dormancy of loblolly pine (Barnett, 1970). Results of the

experiment outlined in Appendix 1 corroborate these findings. In this experiment, seed coats were removed from mature non-stratified seeds and from 35 DAI_2 stratified seeds, and used to enclose either decoated mature non-stratified seeds or 35 DAI_2 stratified seeds following a factorial design. Germination of 35 DAI_2 stratified decoated seeds cultured within mature non-stratified seed coats was only a little lower than that of 35 DAI_2 stratified decoated seeds cultured within 35 DAI_2 stratified seed coats, and nearly identical to that of mature non-stratified decoated seeds cultured within 35 DAI_2 seed coats. The germination of mature non-stratified decoated seeds cultured within mature non-stratified seed coats was lower than that of the other three treatments. If chemical inhibitors of germination were emanating from the mature non-stratified seed coats, then the germination of 35 DAI_2 stratified decoated seeds cultured with mature non-stratified seed coats should have been reduced to that of mature non-stratified decoated seeds cultured with mature non-stratified seed coats, which was not the case. An alternative explanation for this observation may be that stratification renders the decoated seeds less sensitive to any putative inhibitor of germination arising from the seed coat.

Interestingly, germination of mature, non-stratified decoated seeds was reduced more by mature non-stratified seed coats than by 35 DAI_2 stratified seed coats. These data indicate that stratification does alter some parameter(s) of the seed coat which acts as a constraint to germination in the mature seed. Mechanical restriction by the seed coat can be discounted, since the seed coats were split in two along the suture, leaving the abaxial half of the coat physically unconnected to the adaxial half. Gas exchange is also not a factor, because splitting the seed coats in two allowed full gas exchange with the enclosed tissue. A change in the ability of the seed coat to conduct water uptake is

possible, since the decoated seed was completely shielded from the media by the seed coat. This explanation is also consistent with the small difference in germination between 35 DAI_2 stratified decoated seeds cultured within mature non-stratified seed coats and those cultured within 35 DAI_2 stratified seed coats: since the 35 DAI_2 seeds are fully imbibed, restriction of water uptake does not present a barrier to germination. However, because water content of the seeds was not measured in this experiment, this hypothesis cannot be substantiated.

The papery layer that envelops the megagametophyte and nucellar cap reduces the ability of loblolly pine seeds to germinate, as well. Fine structure studies on Norway spruce and Scots pine indicate that the papery layer in these species is composed of remnants of the nucellus, and is lipophilic (Tillman-Sutela and Kauppi, 1995a, 1995b). Like the seed coat, this tissue appears to reduce water and oxygen uptake by the seed in loblolly pine (Barnett, 1976). The papery layer was found to present a significant constraint to germination in the seeds of western white pine (Hoff, 1987), Norway spruce (Tillman-Sutela and Kauppi, 1995a), and Scots pine (Tillman-Sutela and Kauppi, 1995b). In the latter two studies, soluble dyes were used to monitor the course of seed imbibition, rather than by the conventional sequential tissue removal experiments used in this study. This non-intrusive method clearly demonstrated that water uptake was most impeded by the papery layer in Norway spruce and Scots pine.

The nucellar cap decreases the rate of germination in loblolly pine seeds to a small extent: at three days of culture, embryos cultured with an intact megagametophyte demonstrated 95 ± 2% germination, while embryos with an intact megagametophyte and nucellar cap showed only 71 ± 3%. The tissues comprising the nucellar cap are lipophilic,

and thus might reduce the rate of water absorption by the embryo radicle, as has been reported for seeds of Norway spruce (Tillman-Sutela and Kauppi, 1995a) and Scots pine (Tillman-Sutela and Kauppi, 1995b). The tissues at the micropylar tip of the nucellar cap are thickened, and serve to plug the micropylar opening of the megagametophyte, through which the radicle must penetrate to complete germination. Thus, in addition to restricting water uptake, the nucellar cap likely provides mechanical restraint to seedling elongation (Tillman-Sutela and Kauppi, 1995a, 1995b; Downie *et al.*, 1997).

Embryos cultured with intact megagametophytes germinated at nearly the same rate as isolated embryos, indicating that the megagametophyte does not play a significant role in dormancy imposition in loblolly pine seeds.

Endo-β-mannanase, a hemicellulase that breaks down cell wall material, has been associated with the weakening of tissues that impose mechanical constraint to embryo germination (Bewley, 1997). In white spruce, the levels of endo-β-mannanase activity in the embryo, megagametophyte, and nucellar tissues are correlated with weakening of the tissues surrounding the embryo: enzyme activity rises as these tissues weaken, and declines after the seed coats splits along the suture (Downie *et al.*, 1997). Endo-β-mannanases may also play a role in seed germination of loblolly pine, since mechanical restraint imposed by tissues surrounding the embryo appears to present a barrier to embryo germination. However, at least in tomato (*Lycopersicon esculentum* Mill.), the activity of endo-β-mannanase in the endosperm is not sufficient to permit radicle elongation (Toorop *et al.*, 1996). Thus, even if endo-β-mannanase is a factor in overcoming the constraints to germination of loblolly pine seeds, other processes are likely to play a role, as well.

4.2 The Role of Stratification in Post-Embryonic Development

Schneider and Gifford (1994) and Mullen *et al.* (1996) demonstrated that changes in mRNA populations and *in vivo* protein synthesis occur in seeds of loblolly pine during stratification. However, it was not clear whether the process of stratification altered the ability of the embryo to carry out events at the cellular level that are associated with post-embryonic development, given that the germination of isolated embryos excised from mature non-stratified seeds is visually indistinguishable from that of isolated embryos excised from 35 DAI_2 stratified seeds. *In vivo* protein synthesis by isolated mature non-stratified embryos/seedlings and isolated 35 DAI_2 stratified embryos/seedlings at 0 to 3 days in culture indicated that stratification has very little effect on the ability of isolated embryos to synthesize proteins associated with germination and the commencement of seedling growth. Differences observed in the protein profiles between mature non-stratified embryos and 35 DAI_2 stratified embryos at 0 days in culture probably reflect the synthesis of new proteins by the mature embryos in response to imbibition.

Stratification did not affect the ability of isolated seedlings to elicit storage protein breakdown, an event associated with the initial stages of early seedling growth (Stone and Gifford, 1997). Similarly, stratification had little effect on storage reserve breakdown in the megagametophyte (see Section 4.3). These results imply that events that occur during stratification are not prerequisite to reserve mobilization during early seedling growth.

The above discussion argues that changes in gene expression associated with seed stratification play only a subtle role - if any role at all - in preparing the loblolly pine embryo for the changes in protein synthesis required for commencing post-embryonic

growth and development. It would appear that stratification has a greater effect on the seed tissues external to the megagametophyte than on the embryo itself.

4.3 The Breakdown of Protein Reserves in the Megagametophyte and Generation of Free Amino Acid Pools

The 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD phosphate buffer-insoluble proteins represent major storage proteins in both the embryo and the megagametophyte of mature loblolly pine seeds. The data presented in Table 3.1 clearly demonstrate that the majority of the 47-kD, 37.5-kD, 22.5-kD, and 13- to 14-kD proteins are housed in the megagametophyte. Since these proteins represent approximately 70% of the total protein in the mature seed, it is reasonable to assume that the majority of the free amino acids arising from storage protein hydrolysis are generated in the megagametophyte. These buffer-insoluble storage proteins in the megagametophyte are broken down primarily following radicle emergence, which occurs at 4 DAI_{30}. The limited amount of storage protein hydrolysis that takes place prior to radicle emergence indicates that the majority of amino acids arising from storage protein breakdown are used primarily to support metabolic processes that occur during early seedling growth, rather than during dormancy-breaking or germination. These findings are in agreement with those of Gifford *et al.* (1986) in castor bean.

The disappearance of the phosphate buffer-insoluble storage proteins correlated with the increase in soluble amino acids in the megagametophyte, as well as in the seedling. The soluble amino acid pool experiments using cultured seed parts provide several lines of evidence to indicate that the majority of the free amino acids produced in the megagametophyte, predicted to arise from storage protein breakdown, are exported to

the developing seedling. First, the pool of soluble amino acids accumulated by isolated seedlings after 10 days in culture was much smaller than that accumulated by seedlings incubated 10 days in culture with an intact megagametophyte (Fig. 3.11 A). Second, isolated megagametophytes accumulated a far larger pool of soluble amino acids than megagametophytes from intact seeds (Fig. 3.12 A). And third, the megagametophyte was able to export the soluble amino acids that were generated in the absence of the seedling (Fig. 3.12 B), in the form of an exudate that emanates from the cells of the megagametophyte that are adjacent to the corrosion cavity (Fig. 3.13). The ability of isolated megagametophytes to produce an exudate containing the products of reserve breakdown has also been observed in ponderosa pine (Ching, 1970). The relatively small size of the soluble amino acid pool in the megagametophyte from intact seed compared to that of the seedling from intact seed suggests that amino acids are exported by the megagametophyte as they are produced via storage protein hydrolysis, rather than sequestered for later transport.

A preliminary radiotracer experiment conducted with [^{3}H]-leucine supports the hypothesis that amino acids are being exported from the megagametophyte to the seedling (Appendix 2). Further pulse-chase experiments with radiolabelled amino acids are required to confirm these preliminary results. Export of amino acids from the megagametophyte to the seedling is discussed further in Sections 4.6 and 4.8.

4.4 Amino Acid Composition of the Megagametophyte Storage Proteins

Arginine is the major amino acid in the buffer-insoluble storage proteins of loblolly pine, accounting for 23.4 mol% of the amino acids, and nearly half of the nitrogen, comprising these proteins (Table 3.2). Feirer (1995) has also shown that arginine is the

major amino acid in the buffer-insoluble storage proteins of loblolly pine. Arginine is a prominent amino acid in the storage proteins of other conifer species, as well, including maritime pine (Allona *et al.*, 1992, 1994a, 1994c), interior spruce (Newton *et al.*, 1992), Douglas-fir (Leal and Misra, 1993; Feirer, 1995), and eastern white pine (Feirer, 1995). Earlier studies that quantified the amino acid composition of the total protein hydrolysate of the megagametophyte also demonstrated that arginine was a major component comprising the megagametophyte proteins in Italian stone pine (Guitton, 1964) and jack pine (*Pinus banksiana* Lamb.) (Durzan and Chalupa, 1968). The arginine content of the loblolly pine buffer-insoluble proteins is higher than that of the seed proteins in any of the 200 angiosperm species representing 56 families that were surveyed by Van.Etten *et al.* (1963). For example, the *Arabidopsis thaliana* 2S albumin and 12S globulin seed storage proteins are made up of 6.9 and 7.0 mol% arginine, respectively (Zonia *et al.*, 1995), and soybean (*Glycine max* L.) 11S and 7S globulins are comprised of 5.6 and 8.8 mol% arginine, respectively (Derbyshire *et al.*, 1976).

Glutamine and/or glutamate are also present in relatively high proportions in the loblolly pine buffer-insoluble seed storage proteins. The acid hydrolysis technique used in this study did not permit differentiation between glutamine and glutamate. Thus, the proportion of glutamine to glutamate in these proteins cannot be determined from this data. However, even if the most conservative assumption is made that glx is composed entirely of glutamine, yielding two N per molecule of glx, the proportion of N contained in glx is still only half of the N found in the arginine of these storage proteins (Table 3.2). Therefore, arginine would appear to be a more important repository of nitrogen in the major storage proteins in loblolly pine than either glutamine or glutamate.

4.5 Composition of the Free Amino Acid Pools

The breakdown of the arginine-rich storage proteins in the megagametophyte correlates with the large influx of arginine in the free amino acid pools of the megagametophyte, and also of the seedling. Guitton (1964) has presented tenuous data indicating that the increase in free arginine pools in Italian stone pine was the result of proteinase action on seed proteins. High levels of free arginine have been found in both seedlings and megagametophytes of jack pine (Durzan and Chalupa, 1968; Ramaiah *et al.*, 1971) and Italian stone pine (Guitton, 1964) following imbibition, as well as in Scots pine seedlings (Flaig and Mohr, 1992). Although the relative distribution of free arginine in the root pole and shoot pole of the seedlings was not determined in this thesis, studies with Scots pine (Flaig and Mohr, 1992) and jack pine (Durzan and Chalupa, 1968; Ramaiah *et al.*, 1971) indicate that the majority of the free arginine in the seedlings of these species was located in the shoot pole.

Like arginine, the high percentage of glutamine/glutamate in the major storage proteins of the megagametophyte suggests that the breakdown of these proteins might be accompanied by substantial increases in the levels of either or both glutamine and/or glutamate in the megagametophyte and the seedling. The relatively high levels of glutamine in both the seedling and the megagametophyte at 9 DAI_{30} and 12 DAI_{30} support this hypothesis. Glutamine has been demonstrated to be the most abundant amino acid in the xylem sap of mature loblolly pine trees, which indicates that this amino acid may be utilized as a transport compound in loblolly pine (Barnes, 1962). The observed increase in glutamine pool size in the seedling during the latter stages examined could reflect this role. In contrast to glutamine, glutamate is more prevalent in the free amino acid pools prior to

9 DAI_{30}. However, it must be recognized that the free amino acid data presented in this thesis estimate the free amino acid steady-state pool size, and not the rate of flux through amino acid metabolites. Therefore, if the role of free glutamine and/or glutamate arising via storage protein hydrolysis is primarily as metabolic cycle intermediates in a high state of flux, then the importance of these amino acids in loblolly pine early seedling growth may be underrepresented in the steady state data presented in this thesis.

Asparagine is also an important transport amino acid in plants (Lea and Miflin, 1980), and has been demonstrated to be a major amino acid in cotton seedlings, particularly in the vascular exudate (Capdevila and Dure, 1977). The abundance of asparagine in the later stages of seedling growth, contrasting with the relatively low concentrations of asparagine in the non-vascularized megagametophyte, suggests that asparagine may be the form in which amino acid nitrogen is transported from the cotyledons to other parts of the seedling in loblolly pine. Although this hypothesis appears to contradict the work of Barnes (1962), the data presented in that paper were gathered on mature trees, rather than on seedlings still acquiring nutrients from the megagametophyte. Furthermore, environmental conditions such as light and temperature were likely to be very different than those used to grow the seedlings used in this thesis. Radiotracer experiments could be used to verify whether glutamine or asparagine - or even both amino acids - are used as transport compounds during loblolly pine early seedling growth.

4.6 Movement of Amino Acids from the Megagametophyte to the Seedling

In many dicotyledonous species, such as the legumes, the amino acid products of storage protein hydrolysis undergo major metabolic interconversions in the cotyledons

prior to transport to the embryonic axis (Bray, 1983; Lea and Miflin, 1980). However, in cereals, amino acids and small peptides are exported from the endosperm to the developing seedling with very few modifications (Bray, 1983). The substantial rise in the free arginine content of the seedling that coincides with the breakdown of the arginine-rich storage proteins in the megagametophyte raises the possibility that arginine is exported from the megagametophyte to the seedling without conversion to another form for transport. To address this hypothesis, the amino acid composition of the exudate obtained from intact 9 to 10 DAI_{30} megagametophytes cultured without seedlings was analyzed (Table 3.5). Although relatively high quantities of arginine are present in the exudate, the data suggest that glutamine, and not arginine, is the major amino acid transported from the megagametophyte to the seedling. Since the major storage proteins of the megagametophyte contain less glutamine than arginine, the proposed hypothesis does not appear to be sufficient in itself to explain the data. It is possible that a percentage of the arginine arising from storage protein breakdown in the megagametophyte is exported to the seedling, while the remainder is metabolized to other forms in the megagametophyte, either for use in the megagametophyte, or for export to the seedling. It is also possible that glutamine is synthesized *de novo* from glutamate via glutamine synthetase, leading to higher concentrations of glutamine than arginine for export to the seedling. The glutamate for this reaction may also be synthesized *de novo* from other amino acid skeletons; it is conceivable that the carbon skeleton of glutamate, α-ketoglutarate, could be derived from TAG breakdown. Alternatively, the exudate that is produced by the megagametophyte in the absence of the seedling may not accurately reflect the proportions of amino acids transported from the megagametophyte in the intact seed, since export by isolated

megagametophytes occurs in the absence of a sink. To reduce the effect of sink removal, the vacant corrosion cavity could be filled with a high-osmoticum bathing solution, such as that described for analysis of pea seed coats exudates (De Jong and Wolswinkel, 1995), and the exudate collected after a shorter period of time. However, a more rigorous test of the hypothesis that the majority of free arginine in the megagametophyte is exported to the seedling without metabolic interconversion would be provided by radiotracer studies of decoated seedlings using [^{14}C]-arginine.

In seedlings that are larger than 40 mm in length - i.e. from 9 DAI_{30} onwards - the cotyledons are the sole tissue of the seedling making contact with the megagametophyte (data not shown). Thus, during the latter stages of early seedling growth, the cotyledons must be the primary site at which the nutrients exported from the megagametophyte are taken up by the seedling. Prior to 9 DAI_{30}, however, it is unclear whether nutrients exported by the megagametophyte are taken up by all portions of the seedling that are enclosed within the corrosion cavity of the megagametophyte, or whether the cotyledons are the preferential site of import.

4.7 Arginase

4.7.1 The Role of Arginase in Early Seedling Growth

Arginine constitutes a significant proportion of the free amino acid pools of both the megagametophyte and the seedling of loblolly pine during germination and early seedling growth, and as such may be important in the nutrition of the developing seedling. Arginase, which produces ornithine and urea from arginine (Fig. 1.3), has been proposed to be one of the major enzymes participating in the assimilation of arginine into metabolic and biosynthetic pathways in both angiosperm and conifer species (Durzan and Steward,

1983; Polacco and Holland, 1993). Using cell-free extracts, arginase activity was found in the seedling, and to a lesser degree, in the megagametophyte, over the course of early seedling growth. Arginase activity has been measured during the early seedling growth of maritime pine, *Abies pectinata* Lamk. D.C. (Guitton, 1957), Italian stone pine (Guitton, 1964), and Aleppo pine (Citharel and Citharel, 1975). Guitton (1957) also reported arginase activity in the mature seeds of Norway spruce, Scots pine, Douglas-fir, *Pinus laricio* Poir., and *Cedrus atlantica* (Endl.) Manetti. The correlation between the increase in free arginine pools (Fig. 3.10) and arginase activity in the seedling (Fig. 3.15 and Fig 3.16) suggests that arginase also plays a role in arginine metabolism during loblolly pine early seedling growth.

To assess the importance of arginase-mediated arginine breakdown to early seedling growth, experiments were conducted with the urease inhibitor PPD. PPD, a urea analogue, is a tight-binding specific inhibitor of ureases, particularly those of plants (McCarty *et al.*, 1990). If arginase is metabolizing significant quantities of arginine in the seedling during early seedling growth, then an active urease is required to break down urea produced by the arginase reaction. The higher level of urea accumulated by seedlings with intact megagametophytes cultured in the presence of the urease inhibitor PPD compared to seedlings with intact megagametophytes cultured with water (Fig. 3.18) indicates that urease is normally functional in loblolly pine during early seedling growth. Blocking urea breakdown via urease resulted in marked impairment of seedling development (Fig. 3.19), suggesting that nitrogen flow via urea is important for early seedling growth in loblolly pine. However, it is also possible that the toxic properties of

urea were responsible for the observed negative effect of elevated urea pools on seedling growth and development.

The magnitude of difference in urea accumulation by control and PPD-treated seedlings was not as great as that reported in *Arabidopsis* seedlings by Zonia *et al.* (1995), perhaps because arginase activity was considerably lower in PPD-treated seedlings than in control seedlings (Fig. 3.20). These experiments raise the possibility that arginase activity in the seedling could be regulated via feedback inhibition by elevated urea levels. Urea has been shown to greatly reduce the activity of the inducible arginase both *in vitro* and *in vivo* in the lichen *Evernia prunastri* (Legaz and Vicente, 1980; Vicente and Legaz, 1985), although urea did not affect the *in vitro* activity of arginase in Jerusalem artichoke tubers (Wright *et al.*, 1981). Alternatively, downregulation of arginase might be elicited by decreased ammonium levels, as has been reported for the cyanobacterium *Anabaena cycadeae* (Singh and Bisen, 1994). Regulation of arginase is discussed further in Section 4.8.

The localization of most of the seed's arginase activity in the cotyledons of the young seedling implies that the cotyledons are likely to be the primary site of arginine metabolism. This observation is consistent with the hypothesis that the cotyledons are the site at which nutrients exported by the megagametophyte are taken up by the seedling (see Section 4.6). Some activity was also found in the epicotyl, suggesting that arginase-mediated arginine metabolism probably occurs in the growing shoot, as well. It remains to be determined whether the primary role of arginase in the epicotyl is for assimilation of arginine derived from storage reserve breakdown, or if this enzyme functions primarily in general cellular metabolism.

The PPD experiments imply that urea generated by arginine-mediated arginase breakdown is metabolized to ammonium and carbon dioxide by urease (Fig. 1.3). The resulting ammonium would then be available for assimilation into a wide range of nitrogen-containing compounds. Two key enzymes in ammonium assimilation in plants are glutamine synthetase and asparagine synthetase (Oaks, 1994; Sivasankar and Oaks, 1996; Oliveira *et al.*, 1997). Since the levels of both asparagine and glutamine increase concomitantly with the increase in free arginine in the seedling, it is conceivable that ammonium derived from arginine is assimilated into these amino acids. This hypothesis is attractive because one or both of these amino acids are potential nitrogen transport compounds in the loblolly pine seedling (see Section 4.5), and could be tested using radiotracer studies. There is evidence for nitrogen flow from arginine to asparagine during early seedling growth in cotton (Dilworth and Dure, 1978).

In animals, the major form of arginase functions as part of the urea cycle in the liver, which primarily serves to produce urea for excretion of excess nitrogen (Jackson *et al.*, 1986). In contrast, the reactions involved in arginine synthesis (ornithine → arginine) in *Saccharomyces cerevisiae* and *Neurospora crassa* are regulated such that their operation is mutually exclusive from the reactions of arginine catabolism (arginine → ornithine), so that a functional urea cycle does not actually operate in these species as it does in animals (Davis, 1986). Although the possibility that a complete urea cycle is functional during loblolly pine early seedling growth was not addressed in this thesis, weak evidence from studies with various plant species suggest all four reactions of a functional urea cycle may operate in some tissues at some points in development. De Ruiter and Kollöffel (1982) reported coordinate, developmentally regulated patterns of activity for

each of the four enzymes of the urea cycle in developing seeds of *Pisum sativum* L. Durzan (1969) found that in spruce buds at the onset of spring bud burst, [^{14}C]-L-arginine was converted to citrulline via an ornithine intermediate, and that [carbamyl-^{14}C]-L-citrulline was metabolized to argininosuccinate, arginine, and urea, in that order. Micallef and Shelp (1989a) demonstrated that the reaction sequence of ornithine → citrulline → argininosuccinate → arginine occurred in the synthesis of arginine from [ureido-^{14}C]-citrulline and [1-^{14}C]-ornithine applied to developing soybean cotyledons. The same authors also showed that radioactivity from L-[guanidino-^{14}C]-arginine and L-[U-^{14}C] arginine applied to soybean cotyledons at the same stage of development could be recovered in ornithine (Micallef and Shelp, 1989b), although arginine breakdown occurred at a much slower rate than arginine synthesis (Micallef and Shelp, 1989a). The authors proposed that a complete urea cycle is operative in developing soybean cotyledons, and that arginine is both synthesized and degraded via this pathway. However, recent evidence suggests that even though the components for arginine breakdown via arginase are present in developing soybean cotyledons, only the pathway for arginine synthesis is actually operative in the developing tissue under normal conditions. Furthermore, only the catabolic reactions are active *in vivo* in soybean cotyledons during germination (J.C. Polacco, personal communication). Closer examination of the data presented in Micallef and Shelp (1989b) reveals that the radiotracer studies did not show movement of label from one intermediate of the cycle, i.e. arginine, back to the same intermediate. Additionally, Micallef and Shelp applied radiolabelled amino acids by injection, which would likely disrupt intracellular compartments. If spatial separation of the enzymes and/or substrates of arginine metabolism by compartmentalization is a factor in the

regulation of these pathways, then this technique would lead to erroneous conclusions (Polacco and Holland, 1993).

It would seem counterproductive for the cotyledon tissues to produce arginine *de novo* via the biosynthetic reactions of the urea cycle while an apparently large pool of arginine derived from the megagametophyte is available. However, the biosynthetic reactions of the urea cycle can be linked with the TCA cycle, thus providing a point of integration of carbon metabolism with nitrogen metabolism (Bidwell and Durzan, 1975; Durzan and Steward 1983). Such points of integration are requisite for regulating metabolite balance (Foyer *et al.*, 1996) as the products of reserve breakdown are assimilated into metabolic and biosynthetic pathways. Metabolic pathway "cross talk" is also important for the seedling's ability to respond at the cellular level to various environmental cues (Foyer and Galtier, 1996). Thus, argininosuccinate lyase, which converts argininosuccinate to arginine and fumarate (Fig. 1.3), may be active in the cotyledons when conditions favour a net transfer of carbon skeletons from nitrogen metabolic pathways to carbon metabolic pathways. Radiotracer experiments with seedlings cultured under different nutrient or environmental conditions could be employed to test this hypothesis.

4.7.2 Purification and Properties of Arginase

Arginase has been purified to varying degrees in a number of plant species, including pumpkin (Splittstoesser, 1969), Jerusalem artichoke (Wright *et al.*, 1981), iris (Boutin, 1982), and common bean (Carvajal *et al.*, 1996), but has been purified to apparent homogeneity only in soybean (Kang and Cho, 1990). The purification of

arginase to near-homogeneity from loblolly pine shoot poles (Fig. 3.30) represents the first time that this enzyme has been purified from a conifer species.

Arginase from loblolly pine seedling shoot poles demonstrated a dependence on divalent cations for activity (Fig. 3.33). Enzyme activity was restored by addition of Mn^{2+} to the inactivated enzyme, providing evidence that arginase in loblolly pine seedling shoot poles requires a manganese cofactor for activity. These data are corroborated by the observation that arginase activity was diminished when enzyme preparations were subjected to procedures which removed soluble manganese. Preliminary evidence indicated that other divalent cations were not able to restore arginase activity to divalent cation-depleted enzyme preparations (data not shown), which suggests a specificity in the association of manganese with loblolly pine arginase. The requirement of arginase for manganese has been reported in other plant species, including pumpkin (Splittstoesser, 1969), grapes (Roubelakis and Kliewer, 1978), Jerusalem artichoke (Wright *et al.*, 1981), iris bulbs (Boutin, 1982), and common bean (Carvajal *et al.*, 1996). Manganese-dependent arginases are also common in other taxa, including fungi (Davis, 1986; Green *et al.*, 1991b), invertebrates (O'Malley and Terwilliger, 1974), mammals (Hirsch-Kolb and Greenberg, 1968; Vielle-Breitburd and Orth, 1972; Cavalli *et al.*, 1994; Kuhn *et al.*, 1995) and bacteria (Nakamura *et al.*, 1973; Moreno-Vivián *et al.*, 1992; Kanda *et al.*, 1997). The association of manganese clusters with the active site of arginase has been extensively characterized in the rat liver enzyme (Cavalli *et al.*, 1994; Kanyo *et al.*, 1996). However, the metal cofactor requirement of arginases in some species may be more complicated. In *Saccharomyces cerevisiae*, for instance, tightly bound Zn^{2+} ions are required in addition to

the Mn^{2+} ions for maintaining the tertiary and quaternary structure of arginase (Green *et al.*, 1991b).

Multiple peaks corresponding to arginase activity were not detected using any of the chromatographic techniques employed during the purification of arginase, including FPLC gel filtration, FPLC anion exchange, or substrate affinity chromatography. However, the persistent co-chromatography of two bands with similar molecular masses that were resolved using SDS-PAGE (Fig. 3.30) suggests that there may have been two forms of arginase in loblolly pine shoot poles. Alternatively, it is feasible that these bands represented a single subunit that was subject to modification by the process of electrophoresis, resulting in two variants of the same subunit. As an example, the 44 kD protein may have represented arginase subunits which had retained bound manganese during preparation of the sample for SDS-PAGE, while the 43 kD protein may have represented arginase subunits which had lost all bound manganese. Because of the positive charge associated with Mn^{2+}, retention of Mn^{2+} with the arginase subunits would have increased the protein's apparent molecular mass in SDS-PAGE. However, the possibility that either multiple forms of arginase exist *in vivo*, or that the holoprotein is composed up a consistent arrangement of non-identical subunits, cannot be ruled out based upon the available data. Although arginase isoforms have not been reported in higher plants, multiple forms of arginase have been reported in several species from other taxa, including the lichen *Evernia prunastri* (Legaz and Vicente, 1982; Martín-Falquina and Legaz, 1984; Planelles and Legaz, 1987), *Neurospora crassa* (Borkovich and Weiss, 1987a, 1987b), rabbits (Vielle-Breitburd and Orth, 1972), *Rana esculenta* and *Xenopus laevis* (Peiser and Balinsky, 1982), mice (Spolarics and Bond, 1988), rats (Diez *et al.*,

1994), and humans (Zamecka and Porembska, 1988; Vockley *et al.*, 1996). In rat and mouse liver arginases, subunits of two similar but distinct molecular masses appear to comprise the holoprotein (Spolarics and Bond, 1988; Diez *et al.*, 1994). Diez *et al.* (1994) provided strong immunological evidence that the two subunits were antigenically related. It is conceivable that arginase from loblolly pine is similar to liver arginases from rat and mouse liver: however, further investigation is required to substantiate this speculation.

Arginase from loblolly pine shoot poles has a native molecular mass of 140 kD, based upon the mobility of the protein during gel filtration chromatography. The holoprotein is predicted to be a trimer, even though the molecular masses of three subunits, each with an estimated molecular mass of 43 to 44 kD, do not sum to 140 kD. Discrepancies between the estimated holoprotein size and the sum of the subunit masses have been observed for the arginases of other species, including *Neurospora crassa* (Borkovich and Weiss, 1987a). In *Rhodobacter capsulatus*, significantly different estimates for the native molecular mass of arginase were obtained when different techniques were used (Moreno-Vivián *et al.*, 1992), implying that some property of arginase may alter the enzyme's mobility under different conditions. Borkovich and Weiss (1987a) suggested that the shape of the holoprotein is non-spherical, resulting in impeded mobility during gel filtration, although this speculation does not agree with the X-ray diffraction analysis of rat liver arginase (Kanyo *et al.*, 1996). It is possible that the manganese ions associated with the holoprotein - there are two Mn^{2+} per subunit in rat liver arginase (Kanyo *et al.*, 1996) - contribute to the difference between the predicted and actual holoprotein molecular mass.

The arginases of the few plant species that have been examined to date exhibit varied native molecular masses and deduced subunit structure. The arginase from iris bulbs is a 191 kD, hexameric protein (Boutin, 1982), whereas soybean seedling arginase was found to be a tetrameric protein of 240 kD (Kang and Cho, 1990). The native molecular mass of arginase from the tuber of Jerusalem artichoke is 140 kD (Wright *et al.*, 1981), while that of pea cotyledon arginase is 204 kD (Vardanian and Davtian, 1984). Loblolly pine seedling arginase, with a native protein of 140 kD that is composed of 43 to 44 kD subunits, appears to be similar to the liver arginases. The hepatic arginases generally are trimeric proteins of 110 to 140 kD, with subunit molecular masses ranging from 30 to 40 kD (Vielle-Breitburd and Orth, 1972; Zamecka and Porembska, 1988; Cavalli *et al.*, 1994), although the hepatic arginases of some species appear to be tetrameric proteins with native molecular masses similar to the trimeric proteins (Spolarics and Bond, 1988). Some non-hepatic arginases, such as those of *Saccharomyces cerevisiae* and *Rhodobacter capsulatus*, are also trimeric or tetrameric proteins with native molecular masses in the range of the hepatic arginases (Green *et al.*, 1990; Green *et al.*, 1991b; Moreno-Vivián *et al.*, 1992). In contrast, the arginases of other species, such as the polychaete *Pista pacifica* (O'Malley and Terwilliger, 1974), *Neurospora crassa* (Borkovich and Weiss, 1987a), *Bacillus caldovelox* (Bewley *et al.*, 1996), and *Bacillus brevis* (Kanda *et al.*, 1997), are hexameric proteins of approximately 200 kD or greater. It had been postulated that the low molecular weight arginases, which generally have low K_m values, were characteristic of ureotelic species, while the high molecular weight arginases, with comparatively high K_m values, were found in uricotelic species. Physico-chemical differences between the two classes of enzymes were thought to contribute to

different physiological roles (Mora *et al.*, 1965). Although this theory has not held up to close scrutiny (e.g. Reddy and Campbell, 1970; O'Malley and Terwilliger, 1974), classification of arginases as ureotelic or uricotelic still persists in the literature. The biological significance - if any exists - for the differences in native molecular masses and subunit composition of arginases from different species remains to be determined.

Preliminary experiments using washed organellar preparations indicated that arginase in loblolly pine may be associated with organelles (data not shown). Interestingly, the 43 kD protein and the 44 kD protein still co-purified when crude organellar preparations were used as a preliminary step for the arginase purification protocol (data not shown). Enzyme localization studies have been used to demonstrate that arginase is associated with the mitochondria in pea leaves (Taylor and Stewart, 1981), broad bean cotyledons (Kollöffel and van Dijke, 1975), and soybean seedlings (J.C. Polacco, personal communication). Based on biochemical organellar studies, liver arginases were originally proposed to be mitochondrial enzymes in mammals. Subsequent studies have demonstrated that liver arginases are actually tightly associated with the outer mitochondrial membrane via ionic interactions (Spolarics and Bond, 1988), so that these enzymes are actually cytosolic (Jackson *et al.*, 1986). However, non-hepatic arginases from both human and rats have been shown to be mitochondrial enzymes (Gotoh *et al.*, 1996).

The antibodies raised against the denatured arginase subunits appear to be the first to be elicited to a plant arginase; however, further experiments are necessary to conclusively demonstrate that the antibodies are monospecific for arginase. Although the antibodies were raised to both the 43 kD and 44 kD proteins, and the antiserum

recognizes both bands in samples containing equal proportions of these two proteins, the antiserum has a much higher affinity for the lower molecular weight band than the higher molecular weight band in immunoblots of SDS-PAGE buffer-soluble seedling protein profiles. These data suggest that the 44 kD protein is present in lower quantities in the seedling.

4.8 Seedling-Megagametophyte Interactions in Protein Reserve Mobilization and Amino Acid Utilization

The importance of the megagametophyte in providing the products of storage reserve breakdown to the seedling to support growth and development is apparent from the discussion in Sections 4.3 to 4.6. In this section, the roles that interactions between the seedling and the megagametophyte may play in regulating the processes of storage protein mobilization, amino acid export, and arginine utilization are examined.

Megagametophytes cultured either with or without seedlings have similar patterns of storage protein breakdown, indicating that input from the seedling is not required for the megagametophyte to effect storage protein hydrolysis. Furthermore, isolated megagametophytes are able to export the amino acid products of protein reserve breakdown, even so far as not to require a sink for export to occur. These data would seem to indicate that the megagametophyte is autonomous in its ability to carry out the molecular and biochemical events required for storage protein mobilization. However, during the latter stages of culture, storage protein breakdown in isolated megagametophytes virtually ceases, while breakdown of the storage proteins in megagametophytes with seedlings continues (Fig. 3.7). From this observation, it may be inferred that although the megagametophyte has the ability to commence storage protein

mobilization in the absence of the seedling, the seedling is required for the completion of this process.

The accumulation of larger free amino acid pools by isolated megagametophyte halves compared to megagametophytes with intact seedlings (Fig. 3.12 A) indicates that export by the megagametophyte into an artificial sink is not as complete as is export to the seedling. The seedling may be a more efficient sink than agar, since the seedling is closely appressed to the surface of the megagametophyte (Stone and Gifford, 1997), and is able to take up amino acids which are exported by the megagametophyte into the corrosion cavity, effectively removing them from the surface of the megagametophyte. This hypothesis is supported by the observation that breakdown of the storage proteins in the megagametophyte appears to be subject to negative regulation by one of the major products of storage protein hydrolysis, arginine (Fig. 3.8). However, due to the small sample size (n=2), the differences were not statistically significant at $\alpha = 0.05$.

That storage protein breakdown in the megagametophyte could be regulated by levels of free arginine raises the possibility that the seedling may be able to mediate the rate of protein reserve breakdown in the megagametophyte by regulating the rate at which arginine is utilized in the seedling. The marked reduction in storage protein breakdown in megagametophytes of seedlings with intact megagametophytes that were treated with PPD compared to storage protein breakdown in control seedlings with intact megagametophytes (Fig. 3.21) provides circumstantial evidence to support this suggestion. As discussed in Section 4.7.1, blocking urea metabolism in seedlings via urease using the enzyme inhibitor PPD resulted in increased levels of urea, as well as decreased levels of arginase. It is likely that decreased levels of arginase in the seeding

would lead to increased levels of arginine. The elevated levels of arginine in the seedling may have directly mediated a reduction in the export of arginine (and possibly other amino acids) from the megagametophyte, resulting in elevated levels of arginine in the megagametophyte which could then effect a reduction in storage protein breakdown. Alternatively, it is conceivable that the elevated levels of arginine in the PPD-treated seedlings triggered a signaling cascade, resulting in a messenger being exported from the seedling to the megagametophyte to elicit down-regulation of storage protein breakdown.

Together, these data provide some evidence that storage protein hydrolysis is regulated by feedback inhibition by arginine. The effect of arginine and other amino acid products of storage protein breakdown on the regulation of storage protein hydrolysis at the molecular and biochemical level cannot be addressed until specific proteases that are involved in this process are identified. However, exogenously applied amino acids have been shown to reduce the activity of endopeptidases involved in storage protein breakdown in the cotyledons of mung bean (*Vigna mungo*) (Taneyama *et al.*, 1996).

Arginase activity in seedlings from which the megagametophyte was removed prior to or at radicle emergence was greatly reduced (Figs. 3.22 and 2.23), indicating that some input from the megagametophyte is required to induce arginase activity in the seedling. Levels of arginase activity in 9 DAI_{30} seedlings from which the megagametophyte was removed also declined (Fig. 3.24), suggesting that factor(s) from the megagametophyte are also required to maintain arginase activity once expression of the gene encoding arginase has been induced. An attractive hypothesis is that arginine supplied from the megagametophyte acts to induce arginase activity in the seedling. The hypothesis is supported by the observation that arginase activity was greater in 4 DAI_{30} seedlings

without megagametophytes that were cultured with 100 mM arginine than it was in 4 DAI_{30} seedlings without megagametophytes that were cultured without 100 mM arginine (Fig. 3.27). The increase in arginase activity in seedlings cultured with 100 mM arginine was greater than the increase in activity in seedlings cultured with 10 mM arginine, indicating that the response is dose-dependent (data not shown). Arginase activity in isolated 4 DAI_{30} seedlings cultured with arginine was similar to activity levels in seedlings with megagametophytes for the first 10 hours of culture. However, while activity levels increased by 24 hours in 4 DAI_{30} seedlings with megagametophytes, levels fell slightly in isolated 4 DAI_{30} seedlings cultured with arginine. This decline in activity might be attributable to the concomitant rise in urea levels in isolated seedlings cultured with arginine compared to levels in seedlings cultured with megagametophytes (Fig. 3.29), since elevated levels of urea may act to reduce arginase activity (see Section 4.7.1).

Arginine has been shown to induce activity of the inducible arginase *in vivo* in the lichen *Evernia prunastri* (Legaz and Vicente, 1980, 1982), as well as the arginases of *Aspergillus nidulans* (Cybis and Weglenski, 1972), *Rhodobacter capsulatus* (Moreno-Vivián *et al.*, 1992), *Neurospora crassa* and *Saccharomyces cerevisiae* (Davis, 1986).

Interestingly, isolated 9 DAI_{30} seedlings cultured with 100 mM sucrose demonstrated lower levels of arginase activity than isolated 9 DAI_{30} seedlings cultured without sucrose. These experiments raise the possibility that some metabolic pathways - including the arginase-urease pathway - may have been negatively regulated in order to invest available energy and metabolites into the assimilation of the sucrose taken up from the surrounding medium. As mentioned in Section 4.7.1, interactions between carbon and nitrogen utilization pathways play an important role in the seedling's ability to respond to

changes in the nutrient status within the plant, and also to maintain metabolite balance (Chevalier *et al.*, 1996; Foyer *et al.*, 1996). Exogenously-supplied sucrose has been shown to negatively regulate some enzymes involved in nitrogen metabolism in other systems. For example, the accumulation of mRNAs encoding asparagine synthetase is markedly reduced in *Arabidopsis* and *Zea mays* plants cultured with sucrose (Lam *et al.*, 1994; Chevalier *et al.*, 1996). Thus, it is possible that the high levels of sucrose in the media, together with the lack of an exogenously-supplied nitrogen metabolite, resulted in positive regulation of enzymes involved in sucrose metabolism, and negative regulation of enzymes required for amino acid catabolism.

Sucrose had very little effect on arginase activity in 4 DAI_{30} seedlings from which the megagametophyte had been removed (Fig. 3.23), suggesting that these metabolic pathways either are not yet fully functional or are not capable of responding to the mechanism by which metabolites act to regulate the balance between pathways.

4.9 A Model of Storage Protein Mobilization and Amino Acid Utilization During Loblolly Pine Early Seedling Growth

The data and observations outlined in the preceding sections can be summarized in a conceptual model, which is presented in Figure 4.1. The megagametophyte, which is essential for normal seedling growth and development, houses the majority of the storage reserves in the mature seed of loblolly pine. Arginine constitutes the largest percentage of the amino acids comprising the major storage proteins in the megagametophyte, representing nearly half of the nitrogen contained within these proteins. Breakdown of these proteins results in the generation of free amino acid pools, of which arginine is a major component. Arginine may act as a negative regulator of processes involved in

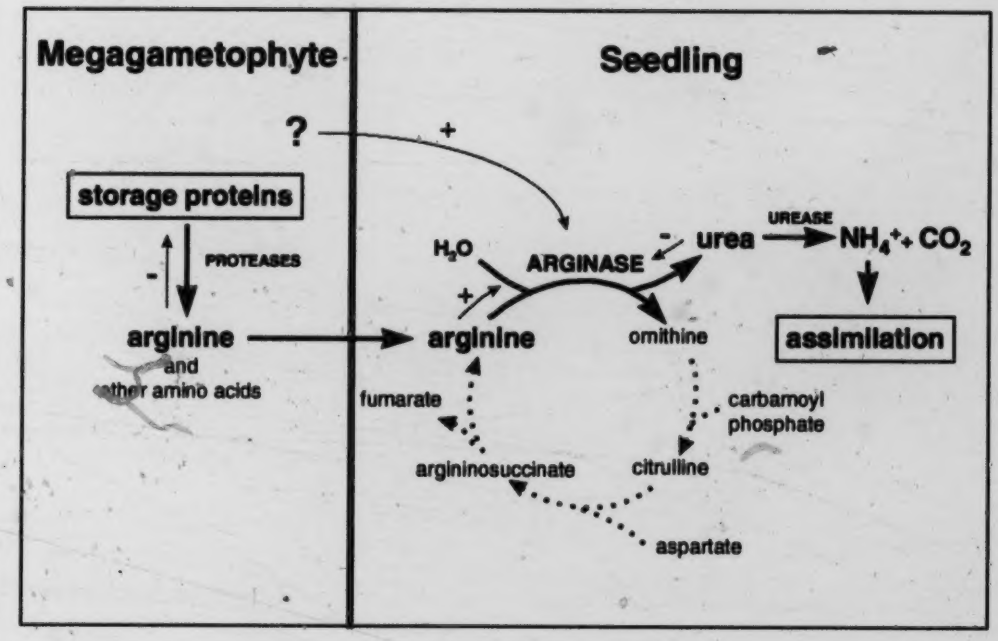


FIGURE 4.1 A conceptual model of storage protein mobilization and amino acid utilization during loblolly pine early seedling growth. The model focuses upon the generation and utilization of arginine, a primary component of the major seed storage proteins in loblolly pine. Black arrows denote the flow of arginine from storage proteins in the megagametophyte to metabolic and biosynthetic pathways in the seedling. Grey arrows accompanied by (+) and (-) signs indicate potential mechanisms regulating these processes.

storage protein hydrolysis. The free amino acids that arise from the breakdown of storage proteins in the megagametophyte are exported to the megagametophyte: preliminary data suggest that at least a proportion of the free arginine generated in the megagametophyte is transported to the seedling without prior conversion to another form for transport. The cotyledons appear to be the site of uptake of metabolites exported from the megagametophyte to the developing seedling, particularly in the latter stages of early seedling growth. Although the megagametophyte does not appear to require a signal from the seedling to elicit the breakdown of storage proteins and export of the resulting amino acids, the seedling is able to modulate these processes to some extent. One possible means by which the seedling could exert some control over storage protein breakdown is via a feedback regulation mechanism mediated by elevated amino acid levels in the seedling.

Assimilation of free arginine into metabolic and biosynthetic pathways in the seedling via the arginase-urease pathway is important for growth and development. Arginase is located primarily in the cotyledons of the developing seedling, and appears to be subject to positive regulation by its substrate, arginine, which is probably supplied to the seedling from the megagametophyte. It is possible that other factors exported from the megagametophyte to the seedling may act to regulate arginase activity, either in a specific manner, or as part of a general mechanism responsible for coordinated up-regulation of gene expression necessary during early seedling growth. Arginase also appears to be subject to negative regulation by one of the products of the reaction, urea, although it is possible that decreased ammonium levels could instead act to down-regulate arginase activity.

4.10 Future Directions

The results presented in this thesis provide some insight into the role of the megagametophyte in the nutrition of the seedling during the early stages of growth and development of loblolly pine, and introduce some novel clues as to how interactions between the megagametophyte and the seedling might mediate the processes of storage protein breakdown and amino acid export and utilization. This information has direct application to seedling production by conventional approaches, as well as via somatic embryogenesis and artificial seed technologies. However, as is often the case, this thesis raises many more questions about the nature of storage reserve mobilization and amino acid utilization than it has answered.

Despite the importance of prompt, synchronous germination to seedling production, our knowledge of the role of stratification in readying seeds of loblolly pine for germination is meagre. The results from this thesis imply that stratification effects changes in the properties of the seed coat that render it more permissive to germination. However, these properties have yet to be identified. The details of how these barriers to germination are altered by stratification need to be determined, as well.

The studies that examined the breakdown of the major storage proteins in the megagametophyte identified some factors that may play a role in regulating storage protein breakdown. However, none of the enzymes involved in storage protein proteolysis in loblolly pine have been identified to date, so the effect of these factors on storage protein breakdown could only be examined in a descriptive fashion. Until the means are available to quantify specific endopeptidases that act to break down storage proteins in the megagametophyte, the processes involved in storage protein hydrolysis,

and the mechanisms by which these processes are regulated, will remain obscure. Thus, an effort should be made to identify and characterize endopeptidases in loblolly pine that participate in storage protein hydrolysis, and to clone cDNAs that code for these enzymes.

Many of the conclusions that have been reached about the fate of arginine that arises from storage protein breakdown have been based upon correlative, rather than direct, evidence. It is essential that the fate of free arginine in the megagametophyte be further investigated using radiotracer studies. Pulse-chase experiments in which [^{14}C]-arginine is applied to the megagametophyte of a decoated seedling as described in Appendix 2 are necessary to determine the proportion of the arginine pool in the megagametophyte that is exported to the seedling without metabolic interconversion to another form for transport. Pulse-chase experiments with [^{14}C]-arginine, as well as *in vivo* [^{15}N]-arginine labelling studies, are also required to trace the metabolic fate of the carbon skeleton and nitrogen moieties of free arginine in both the seedling and the megagametophyte during early seedling growth. These investigations will determine the relative flux of arginine through the arginase-urease pathway in the cotyledons, thus providing a more direct measure of the importance of this metabolic route in the utilization of arginine arising from storage protein breakdown. The radiotracer studies will also indicate other significant metabolic routes by which arginine is metabolized during early seedling growth, so that these pathways may be studied, as well. With the exception of polyamine biosynthesis, very little is known about the alternative routes of arginase catabolism in plants. Pulse-chase radiotracer studies could also be used to determine whether a functional urea cycle is operative in loblolly pine during early seedling growth.

The antibodies that have been raised to loblolly pine arginase appear to be the first antibodies elicited to a plant arginase, and will be a pivotal component of future investigations in this laboratory examining the developmental regulation of arginase during early seedling growth, and perhaps during other phases of the life cycle, as well. These immunological studies of protein levels should be augmented with studies of arginase gene expression, which will require isolation of a cDNA clone encoding arginase. This can now be accomplished by screening an expression cDNA library that has been synthesized from loblolly pine shoot poles (Mullen, 1995) with the anti-arginase antibodies. The sequence of the cDNA clone, together with an N-terminal sequence for the purified protein, would verify that the proteins to which the antibodies were raised represent arginase. A comparison of enzyme activity levels in cell-free extracts with steady state mRNA and protein levels over the course of early seedling growth will provide some information on mechanisms by which arginase gene expression is developmentally regulated. The availability of antibody and cDNA probes will also allow for a more thorough investigation of how the megagametophyte acts to regulate arginase in the seedling. The effect of factors such as arginine, urea, and other metabolites on levels of arginase mRNA, protein, and activity levels can also be ascertained, in order to understand the level at which these factors act to modulate arginase gene expression. Together, these studies will advance our knowledge of how nutrient utilization is regulated during early seedling growth in loblolly pine.

In addition to the studies focusing upon the regulation of arginase, the antibodies can be used for immunolocalization studies to determine the tissue distribution of this enzyme, as well as its intracellular compartmentalization. A comparison of

immunoprecipitation products of arginase synthesized *in vivo* and arginase synthesized from mRNA *in vitro* may reveal post-translational proteolytic processing that is indicative of mitochondrial transit sequence cleavage. Since compartmentalization has been shown to be an important factor in the regulating arginine flux through the arginase-urease pathway in other organisms, knowledge about the cellular location of arginase may indicate whether compartmentalization plays a role in the regulation of arginase-mediated arginine catabolism in loblolly pine. Finally, these antibodies will also provide a useful tool for immunological comparisons of plant arginases, particularly those from other conifer species.

The studies described in this thesis that examined seedling-megagametophyte relationships did not address whether the megagametophyte simply acts as source of nutrients for early seedling growth, or whether the megagametophyte also exports growth regulating substances or other signals that direct seedling growth and development. It is imperative that an extensive study be conducted on the composition of the chemical milieu exported from the megagametophyte to the seedling at different points during germination and early seedling growth. These studies would entail examination of the megagametophyte exudate, as well as radiotracer studies. Such experiments will not only provide essential quantitative information on the nutrients that the megagametophyte provides to the seedling, but may also identify growth regulating substances by which the megagametophyte is able modulate processes that occur in the seedling.

Similarly, only indirect evidence on the role that the seedling might play in regulating storage protein mobilization in the megagametophyte was presented in this thesis. More in-depth studies using radiolabelled amino acids and enzyme inhibitors are

required to test the hypothesis that the seedling can bring about end product repression of storage protein breakdown in the megagametophyte, and thus elicit some control over the supply of nutrients that it receives from the megagametophyte.

In summary, the studies presented in this thesis on storage protein breakdown and the generation of free amino acid pools, together with the studies on the utilization of arginine, a major product of storage protein breakdown, have been used to generate a conceptual model of the processes involved in this chain of events. This model lays a solid foundation for more comprehensive investigations into the transport and metabolism of arginine arising from storage protein breakdown - as well as the mechanisms by which these processes are regulated - during loblolly pine early seedling growth.

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APPENDIX 1

Effect of Stratification on the Efficacy of the Seed Coat in Preventing Seed Germination

Rationale

The results presented in Section 3.1 indicated that the seed coat presents the major constraint to germination in loblolly pine. However, these experiments did not identify the mechanism by which the seed coat acts to inhibit the germination of non-stratified seeds. The experiment described in this Appendix was based upon a suggestion in Lang (1994), and was designed to test whether inhibitors emanating from the seed coat may be a factor in the coat-imposed dormancy exhibited by loblolly pine seeds. This experimental design provides a stronger test of the hypothesis than the seed coat leachate experiments conducted by Barnett (1970).

Materials and Methods

Using aseptic technique, seed coats and papery layers were removed from mature non-stratified and 35 DAI_2 stratified seeds. Mature non-stratified and 35 DAI_2 stratified decoated seeds were then enclosed within the two halves of seed coats that were removed from either mature non-stratified and 35 DAI_2 stratified seeds, using a factorial design. Seed coats were not used to envelope the same seed from which they were removed. The decoated seeds with enveloping seed coats were placed in Petri dishes on Kimpak moistened with sterile water, and incubated at 30°C under constant 19 $\mu mol\ m^{-2}\ s^{-1}$ light.

Germination was monitored daily, and was considered to be complete when the radicle had protruded beyond the edge of the seed coat.

Results

The percent germination of 35 DAI_2 stratified decoated seeds was lower if the seeds were enclosed within seed coats removed from a mature, non-stratified seed, rather than those from 35 DAI_2 stratified seeds (Fig. A1). The percent germination of mature, non-stratified decoated seeds enclosed within 35 DAI_2 stratified seed coats was nearly identical to that of the 35 DAI_2 stratified seeds enclosed within mature, non-stratified seed coats. The percent germination of mature, non-stratified decoated seeds enclosed within mature, non-stratified seed coats was lower than that the other three treatments. ANOVA analysis demonstrated that there were significant differences between the treatments at 3 days of culture, at α=0.05.

The significance of these data are discussed in Section 4.1 of the thesis.

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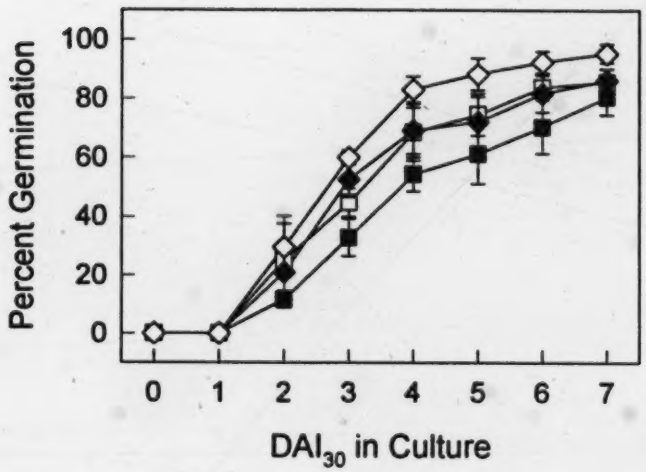

FIGURE A1 Effect of non-stratified and stratified seed coats on percent germination of non-stratified and stratified decoated seeds. Mature, non-stratified decoated seed enclosed within a seed coat from a mature, non-stratified seed (■); mature, non-stratified decoated seed enclosed within a seed coat from a 35 DAI_2 stratified seed (□); 35 DAI_2 stratified decoated seed enclosed within a seed coat from a mature, non-stratified seed (◆); 35 DAI_2 stratified decoated seed enclosed within a seed coat from a 35 DAI_2 stratified seed (◇). Each data point represents the mean percent germination ± SE of four separate experiments, with 20 to 70 individuals per treatment per experiment.

APPENDIX 2

Movement of [^{3}H]-Leucine from the Megagametophyte to the Seedling

Rationale

Radiotracer experiments would provide the best test of the hypothesis that arginine and other products of storage reserve breakdown are transported from the megagametophyte to the seedling. The purpose of the experiment described in this Appendix was to develop a method by which radiolabelled compounds could be applied to megagametophytes with intact seedlings in a relatively non-intrusive method, and to test the hypothesis that radiolabelled compounds applied to the megagametophyte can be transported to the seedling.

Materials and Methods

Seed coats were removed from 10 to 11 DAI_{30} seedlings, and a sharp razor blade used to remove a very thin layer of cells from the thickest part of the megagametophyte, in parallel with the corrosion cavity. The megagametophyte (with intact seedling) was then inserted into a small vessel, so that the slice was placed in contact with approximately 0.8 µCi of [^{3}H]-leu in a total volume of 10 µL. Care was taken that no part of the seedling could make contact with the radiolabelled substrate. The seedlings were then placed in Petri dishes.

Seedlings were pulse-labelled at 30°C in 19 µmol m^{-2} s^{-1} light for 2 to 12 h with [^{3}H]-leu. Two independent replicates of five seed parts each were labelled per treatment. Following incubation, the megagametophytes were washed carefully and thoroughly to

remove excess [^{3}H]-leu. Seed parts were extracted in 1 mL 50 mM sodium phosphate buffer (pH 7.5), then microcentrifuged at 16 000 g (14 000 rpm) in an Eppendorf 5415 Centrifuge (Brinkmann, Westbury, NY). The radioactivity of the supernatant was determined as dpm on duplicate samples for each replicate with a Beckman LS6000TA scintillation counter (Missisauga, ON), using Amersham (Oakville, ON) ACS aqueous liquid scintillant.

Results and Discussion

After 2 h, radioactivity was detected in both the seedling and the megagametophyte. While the level of radioactivity in the megagametophyte remained relatively constant from 2 to 12 h of labelling, the level of radioactivity in the seedling increased by approximately 46% (Table A1). Therefore, radiolabel applied to the megagametophyte appears to have been exported from the megagametophyte to the seedling. However, it cannot be determined from the data the forms in which the ^{3}H was exported.

The method outlined above provides a useful starting point for future experiments examining the movement of metabolites from the megagametophyte to the seedling.

TABLE A1 Movement of [^{3}H]-Leucine Applied to the Megagametophyte of Decoated Seeds.

Time	Radioactivity per Seed Part (dpm x 10^{-3})	
	Megagametophyte	Seedling
2 h	326.8 ± 19.2	169.7 ± 0.5
4 h	319.0 ± 2.1	196.2 ± 10.5
8 h	316.3 ± 9.1	218.1 ± 28.2
12 h	318.5 ± 9.9	248.3 ± 10.2

[a]Values are the mean ± SE of two independent replicates, sampled in duplicate.

APPENDIX 3

Optimization of the Assay for Arginase Activity

Rationale

Optimization of enzyme assays is a necessary prerequisite to data collection. In this thesis, arginase activity was determined by quantifying the amount of urea generated by a cell-free extract incubated with excess arginine at 30°C. The urea was quantified by the carbamido-diacetyl monoxime reaction (Fearon, 1939; Coulombe and Favreau, 1963). Five parameters were examined: time of incubation of the cell-free extract with arginine, the pH optimum of the assay, the presence of endogenous urea in the cell-free extracts, the linearity of the of urea standard curve, and the presence of active ureases in the cell-free extract.

Materials and Methods

For each replicate, five seedlings were extracted in 1 mL extraction buffer [0.1 M Tris·HCl, 0.5 mM $MnSO_4$, 0.75 mM maleate (pH 9.15)] as described by Martin-Falquina and Legaz (1984). The enzyme was activated by pre-incubating extracts for 60 min at 30°C prior to assay. Enzyme activity was assayed by incubating 100 µL of the activated extract with 0.285 M arginine (pH 9.7), 1 mM $MnSO_4$, and 1 mM maleate in a total volume of 1.5 mL for 30 min at 30°C; the reaction was terminated with 0.7 vol 87% (v/v) acetic acid (Greenberg, 1955). Urea production was measured in triplicate by the carbamido-diacetyl monoxime colorimetric method described in Geyer and Dabich (1971). All assay chemicals were purchased from Sigma (St. Louis, MO).

In experiments testing for the presence of an active urease, paired extracts were incubated either in the presence or absence of 50 mM phenylphosphorodiamidate (PPD) (ICN, Costa Mesa, CA) for one hour prior to assaying enzyme activity, according to Zonia *et al.* (1995).

Results and Discussion

Determination of the Linear Portion of the Enzyme Assay

Urea generation by cell-free extracts of 8 DAI_{30} seedlings was linear for at least 30 min at 30°C (Fig. A2). Hence, the assays for enzyme activity were performed for 30 min at 30°C.

pH Optimum of the Enzyme Assay

The activity of arginase in cell-free extracts of 12 DAI_{30} seedlings exhibited a broad pH range in which it was active (Fig. A3). The pH optimum fell between pH 9.5 and 10.0: therefore, pH 9.7 was chosen for use in all subsequent assays.

Endogenous Urea in the Cell-Free Extracts

Extracts from both seedlings and megagametophytes that were first boiled to inactivate any enzyme activity, then assayed as described in Materials and Methods, contained compounds that produced measurable levels of colour reagent in the urea determination assay. These compounds were assumed to be urea: however, since no attempt was made to determine which chemicals other than urea reacted with the assay reagents in the colorimetric assay, these endogenous compounds are referred to as "urea equivalents" in this thesis. Therefore, a boiled control was included for each sample that was analyzed, so that the endogenous urea equivalents could be subtracted from the total

urea that was determined by the colorimetric assay, to obtain a more accurate measure of the urea generated during the enzyme assay. The urea equivalents data for intact seed are illustrated in Figure 3.17 in the body of the thesis.

Linearity of the Urea Standard Curve

The absorbance value of the coloured compound produced by the reaction of urea with thiosemicarbazide and 2,3-butanedione monoxime was linear to approximately 20 µg urea per reaction tube (Fig. A4). However, to stay within the bounds of the linear portion of this curve, urea concentrations from 1 to 15 µg were used to construct the standard curves that were included with every assay. The r^2 values for these standards curves were always greater than 0.99 (data not shown).

Detection of Active Ureases in the Enzyme Assay Mixture

The levels of arginase activity in cell-free crude extracts that were incubated with or without PPD were not significantly different at α=0.05, for both mature embryos or 12 DAI_{30} seedlings (Fig. A5). Thus, urease appears not to have been active under the conditions used for the assay. These results also demonstrate that arginase activity is not significantly affected by PPD.

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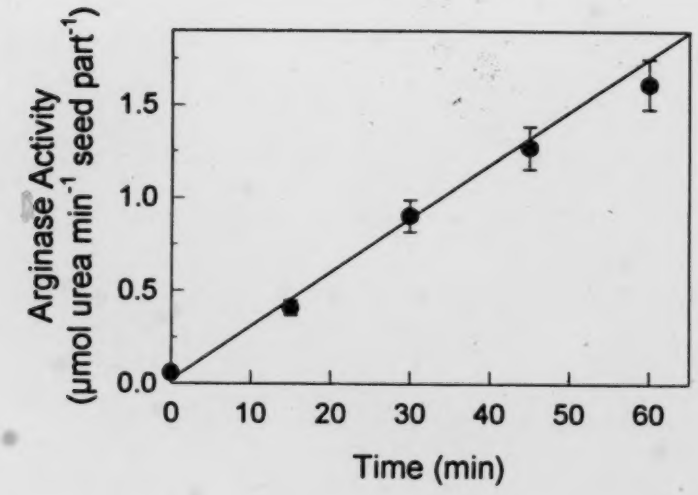

FIGURE A2 Time course of urea generation by cell-free crude extracts of 8 DAI_{30} seedlings. Values represent the mean ± SE of three separate determinations, analyzed in triplicate.

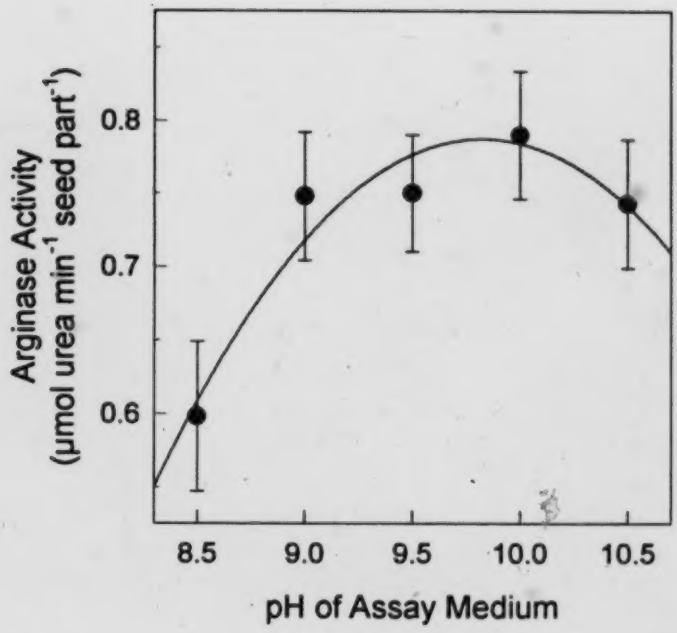

FIGURE A3 Determination of pH optimum for the assay of arginase activity from cell-free crude extracts of 12 DAI_{30} seedlings. Values represent the mean ± SE of four independent determinations, analyzed in triplicate.

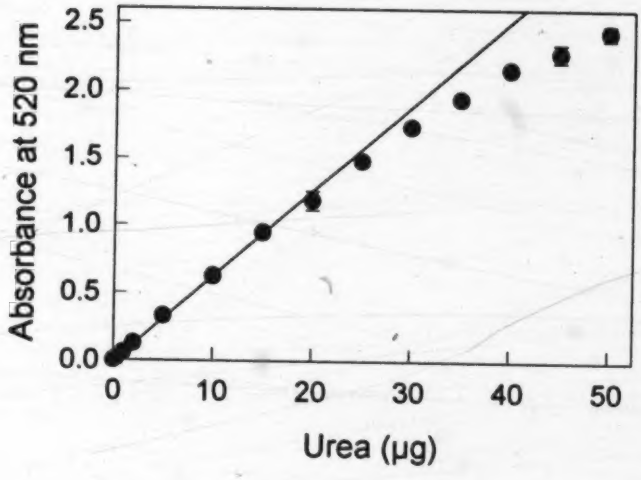

FIGURE A4 The urea curve used to determine the appropriate range of urea concentrations for the standard curves used in the urea colorimetric assay. Values represent the mean ± SD of two independent determinations.

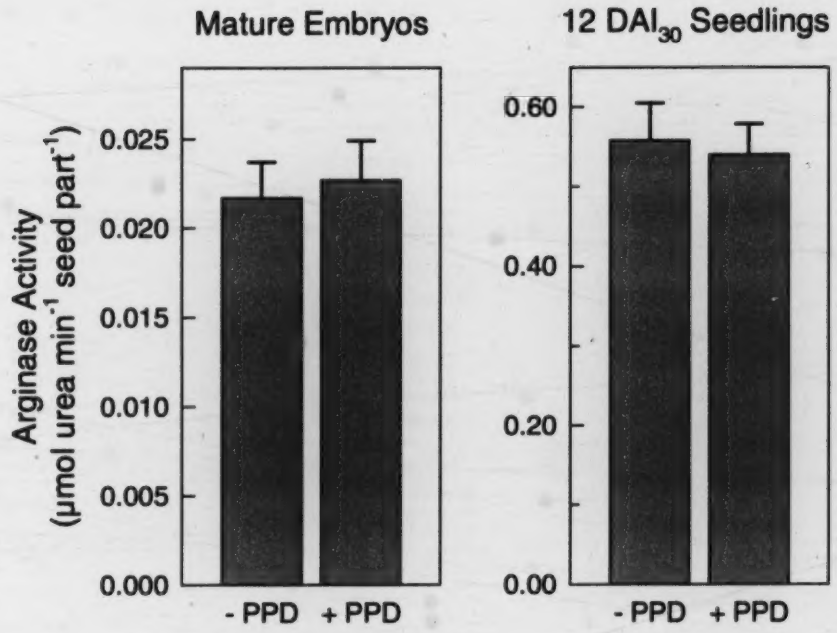

FIGURE A5 Effect of the urease inhibitor PPD on the determination of arginase activity from cell-free crude extracts of mature embryos and 12 DAI_{30} seedlings. Values are a mean ± SE of six independent replicates for the mature embryo, and three independent replicates for the 12 DAI_{30} seedlings.